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Sequence-specific DNA Damage Induced by Carcinogenic Danthron and Anthraquinone in the Presence of Cu(II), Cytochrome P450 Reductase and NADPH

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The mechanism of metal-mediated DNA damage by carcinogenic danthron (1,8-dihydroxyanthraquinone) and anthraquinone was investigated by the DNA sequencing technique using ^{32}P-labeled human DNA fragments obtained from the human c-Ha-***ras***-1 proto-oncogene and the ***p53*** tumor suppressor gene. Danthron caused DNA damage particularly at guanines in the 5′-GG-3′, 5′-GGGG-3′, 5′-GGGGG-3′ sequences (damaged bases are underlined) in the presence of Cu(II), cytochrome P450 reductase and the NADPH-generating system. The DNA damage was inhibited by catalase and bathocuproine, suggesting the involvement of H_2O_2 and Cu(I). The formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine increased with increasing concentration of danthron. On the other hand, carcinogenic anthraquinone induced less oxidative DNA damage than danthron. Electron spin resonance study showed that the semiquinone radical could be produced by P450 reductase plus NADPH-mediated reduction of danthron, while little signal was observed with anthraquinone. These results suggest that danthron is much more likely to be reduced by P450 reductase and generate reactive oxygen species through the redox cycle, leading to more extensive Cu(II)-mediated DNA damage than anthraquinone. In the case of anthraquinone, its hydroxylated metabolites with similar reactivity to danthron may participate in DNA damage ***in vivo***. We conclude that oxidative DNA damage by danthron and anthraquinone seems to be relevant for the expression of their carcinogenicity.

Keywords: Oxidative DNA damage, danthron, anthraquinone, NADPH, copper, P450 reductase

Abbreviations: DTPA, diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid; ESR, electron spin resonance; H_2O_2, hydrogen peroxide; HPLC, high-performance liquid chromatography; HPLC-ECD, HPLC equipped with an electrochemical detector; NADPH, β-nicotinamide adenine dinucleotide phosphate (reduced form); $NADP^+$, β-nicotinamide adenine dinucleotide phosphate (oxidized form); O_2^-, superoxide anion radical; •OH, free hydroxyl radical; 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; SOD, superoxide dismutase

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INTRODUCTION

A derivative of anthraquinone, danthron, has been widely used as a laxative and an intermediate for dyes.[1] There is sufficient evidence for the carcinogenicity of danthron in experimental animals: hepatocellular carcinomas in mice,[2] and adenocarcinoma of the colon in rats.[3] The International Agency for Research on Cancer (IARC) has classified danthron as a group 2B carcinogen,[1] which is possibly carcinogenic to humans. Anthraquinone is widely used as an intermediate in the manufacture of dyes and pigments.[4] The available data were adequate to evaluate the carcinogenicity of anthraquinone in experimental animals.[4] However, the mechanism of DNA damage induced by anthraquinones remains to be clarified.

Recently, danthron has been reported to have genotoxicity to mammalian cells which was attributed to their inhibitory effects on topoisomerase II.[5] However, a variety of quinone derivatives are highly redox active molecules capable of causing oxidative DNA damage through the generation of reactive oxygen species.[6–13] Therefore, in this study, we examined whether anthraquinones (danthron and anthraquinone) could cause oxidative DNA damage in the presence of metal ion, cytochrome P450 reductase and the NADPH-generating system using ^{32}P-5′-end-labeled DNA fragments obtained from the c-Ha-*ras*-1 and the *p53* genes. We measured the content of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) in calf thymus DNA using a high performance liquid chromatograph equipped with an electrochemical detector (HPLC-ECD). Furthermore, we examined the generation of radicals from anthraquinones with an electron spin resonance (ESR) spectrometer.

MATERIALS AND METHODS

Materials

Restriction enzymes (*Apa*I, *Ava*I, *Eco*RI, *Pst*I and *Xba*I) and T_4 polynucleotide kinase were purchased from New England Biolabs. Calf intestine phosphatase and glucose-6-phosphate-dehydrogenase were from Boehringer Manheim GmbH. [γ-^{32}P]ATP (222 TBq/mmol) was from New England Nuclear. Anthraquinones (danthron and anthraquinone) were from Aldrich Chemical Co. Diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (DTPA) and bathocuproinedisulfonic acid were from Dojin Chemicals Co. (Kumamoto, Japan). Reduced form of β-nicotinamide adenine dinucleotide phosphate (NADPH), and its oxidized form ($NADP^+$), acrylamide, bisacrylamide, piperidine and hydrogen peroxide (H_2O_2) were from Wako Chemicals Co. (Osaka, Japan). $CuCl_2$, $MgCl_2$, ethanol and D-mannitol were from Nacalai Tesque, Inc. (Kyoto, Japan). Calf thymus DNA, superoxide dismutase (SOD, 3000 units/mg from bovine erythrocytes) and catalase (45000 units/mg from bovine liver) were from Sigma Chemical Co. Nuclease P_1 was from Yamasa Shoyu Co. (Chiba, Japan). Cytochrome P450 reductase (10.0 mg/ml protein from human microsomes) was from Gentest Corporation.

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

DNA fragments were obtained from the human *p53* tumor suppressor gene [14] and the c-Ha-*ras*-1 protooncogene.[15] The DNA fragment of the *p53* tumor suppressor gene was prepared from pUC18 plasmid. The singly ^{32}P-5′-end-labeled 443-base pair fragment (*Apa*I 14179-*Eco*RI* 14621) was obtained according to the method described previously.[16] 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.[17,18] The singly labeled 341-base pair fragment (*Xba*I 1906-*Ava*I* 2246) and 98-base pair fragment (*Ava*I* 2247-*Pst*I 2344) were obtained according to the method described previously.[17,18] The asterisk indicates ^{32}P-labeling.

Detection of Damage to Isolated DNA

The standard reaction mixtures in a 1.5-ml microtube containing danthron or anthraquinone, 2.5 μg/ml cytochrome P450 reductase, 250 μM $NADP^+$, 500 μM glucose-6-phosphate, 0.1 units/ml glucose-6-phosphate dehydrogenase and 500 μM $MgCl_2$ in 200 μl of 10 mM bicarbonate buffer (pH 7.8) containing 5 μM DTPA were incubated for 30 min at 37 °C. And then, ^{32}P-5′-end-labeled DNA fragment, 2 μM/base calf thymus DNA and 20 μM $CuCl_2$ were added to the mixtures, followed by the incubation for 120 min at 37 °C. The DNA fragments were treated with 1 M piperidine for 20 min at 90 °C where indicated and electrophoresed as described previously.[17] The experiments were performed in air-saturated solution.

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[19] using a DNA-sequencing system (LKB 2010 Macrophor). A laser densitometer (LKB 2222 UltroScan XL) was used for the measurement of the relative amounts of oligonucleotides from treated DNA fragments.

Measurement of 8-oxodG Formation in Calf Thymus DNA

The amount of 8-oxodG was measured by a modified method of Kasai *et al.*[20] The standard reaction mixtures containing danthron or anthraquinone, 2.5 μg/ml cytochrome P450 reductase, 250 μM $NADP^+$, 500 μM glucose-6-phosphate, 0.1 units/ml glucose-6-phosphate dehydrogenase and 500 μM $MgCl_2$ in 10 mM bicarbonate buffer (pH 7.8) containing 5 μM DTPA were incubated for 30 min at 37 °C. And then, 100 μM/base calf thymus DNA and 20 μM $CuCl_2$ were added to the mixtures, followed by the incubation for 120 min at 37 °C. To stop the reaction, 0.2 mM DTPA was added. After ethanol precipitation, the DNA was digested to the nucleosides by incubation with nuclease P_1 and alkaline phosphatase and analyzed with an HPLC-ECD as described previously.[21]

ESR Spectra Measurements

ESR spectra were measured at room temperature (25 °C) by using a JES-TE-100 (JEOL, Tokyo, Japan) spectrometer with 100-kHz field modulation according to the method described previously.[22] Spectra were recorded with microwave power of 2 mW, modulation amplitude of 0.050 mT, receiver gain of 200, time constant 0.3 sec and sweep time 8 min. The magnetic fields were calculated by the splitting of Mn(II) in MgO ($\Delta H_{3-4} = 8.69$ mT).

RESULTS

DNA Damage by Anthraquinones in the Presence of Cytochrome P450 Reductase, the NADPH-generating System and Metal Ions

Figure 1 shows the DNA damage induced by anthraquinone or danthron in the presence of cytochrome P450 reductase, the NADPH-generating system and Cu(II). DNA damage was not observed in the absence of cytochrome P450 reductase (data not shown). The intensity of DNA damage increased depending on their concentrations. Danthron induced DNA damage at much lower concentrations than anthraquinone. The DNA damage was enhanced by piperidine treatment (data not shown), suggesting that these anthraquinones caused not only backbone breakage but also base damage. Danthron and anthraquinone caused the DNA damage in the presence of Cu(II), but not in the presence of Fe(II), Fe(III) or Mn(II) (data not shown).

Effects of Scavengers and Metal Chelators on DNA Damage by Danthron

Figure 2 shows the effects of scavengers and bathocuproine, a Cu(I)-specific chelator, on DNA

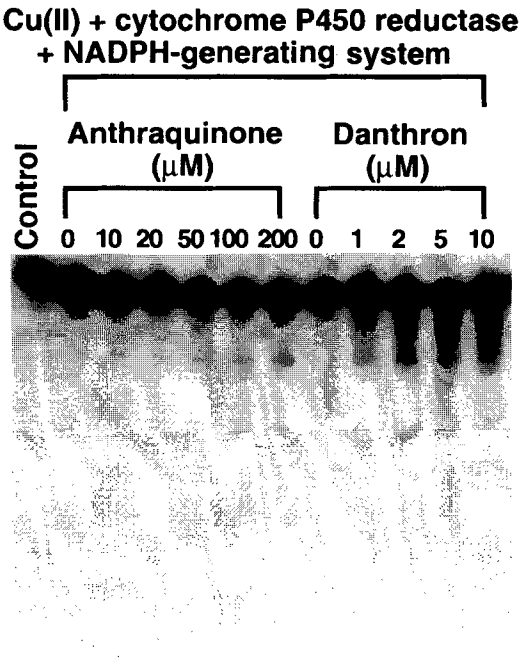

FIGURE 1 DNA damage by anthraquinones in the presence of Cu(II), cytochrome P450 reductase and the NADPH-generating system. The reaction mixtures containing danthron or anthraquinone, 2.5 μg/ml cytochrome P450 reductase, 250 μM $NADP^+$, 500 μM glucose-6-phosphate, 0.1 units/ml glucose-6-phosphate dehydrogenase and 500 μM $MgCl_2$ in 200 μl of 10 mM bicarbonate buffer (pH 7.8) containing 5 μM DTPA were incubated for 30 min at 37 °C. Subsequently, the ^{32}P-5′-end-labeled 98-base pair DNA fragment, 2 μM/base of calf thymus DNA and 20 μM $CuCl_2$ were added to the mixtures, followed by the incubation for 120 min at 37 °C. The DNA fragments were treated with 1 M piperidine for 20 min at 90 °C, and electrophoresed on an 8% polyacrylamide/8 M urea gel. The autoradiogram was obtained by exposing an X-ray film to the gel. The control contained none of anthraquinones, Cu(II), cytochrome P450 reductase and the NADPH-generating system.

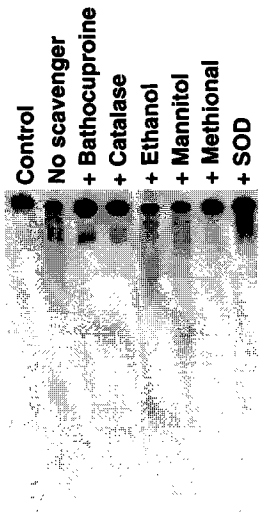

FIGURE 2 Effects of scavengers and bathocuproine on DNA damage induced by danthron. The reaction mixtures containing 50 μM danthron, 2.5 μg/ml cytochrome P450 reductase, 250 μM $NADP^+$, 500 μM glucose-6-phosphate, 0.1 units/ml glucose-6-phosphate dehydrogenase and 500 μM $MgCl_2$ in 200 μl of 10 mM bicarbonate buffer (pH 7.8) containing 5 μM DTPA were incubated 30 min at 37 °C. Subsequently, the ^{32}P-5′-end-labeled 341-base pair DNA fragment, 10 μM/base of calf thymus DNA and 20 μM $CuCl_2$ were added to the mixtures, followed by the incubation for 120 min at 37 °C. The DNA fragments were treated by the method described in the legend to Figure 1. The control contained none of anthraquinones, Cu(II), cytochrome P450 reductase and the NADPH-generating system. Where indicated, 50 μM bathocuproine, 500 units/ml of catalase, 10% ethanol, 0.1 M mannitol, 0.1 M methional or 100 units/ml of SOD was added.

damage induced by danthron in the presence of cytochrome P450 reductase, the NADPH-generating system and Cu(II). Bathocuproine and catalase inhibited DNA damage, suggesting the involvement of Cu(I) and H_2O_2. DTPA also inhibited DNA damage (data not shown). Methional, which scavenges not only •OH but also crypto-OH radicals,[23,24] completely inhibited the DNA damage, although other typical hydroxyl radical (•OH) scavengers (ethanol and mannitol) did not inhibit DNA damage. SOD showed a little inhibitory effect on DNA damage, but not completely. Similar results were obtained for anthraquinone (data not shown).

Site Specificity of DNA Damage by Anthraquinones

Figures 3 and 4 show the site specificity of DNA damage induced by danthron and anthraquinone. Danthron caused preferential DNA damage at the 5′-GG-3′, 5′-GGGG-3′ and 5′-GGGGG-3′ sequences (damaged bases are underlined) in the human c-Ha-*ras*-1 protooncogene (Figure 3A) and the *p53* tumor suppressor gene (Figure 4A-1). In addition, danthron caused DNA cleavage at the guanine residue of the 5′-ACG-3′ sequence (Figure 4A-1). It is interesting that the

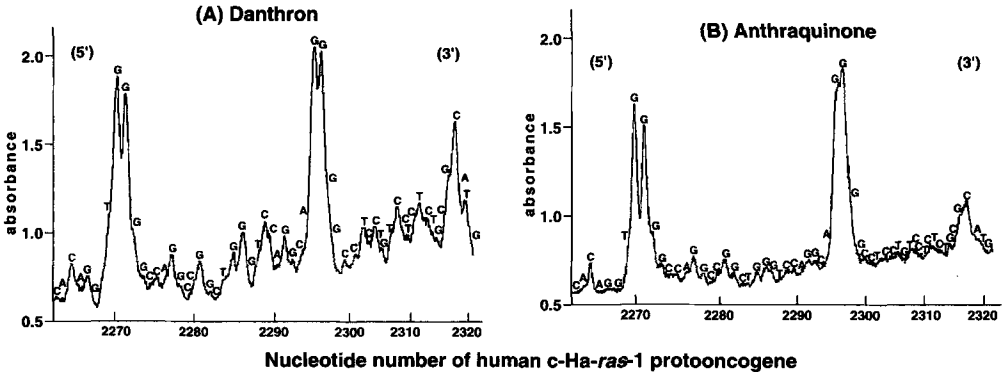

FIGURE 3 Site specificity of DNA damage by anthraquinones. The reaction mixtures containing 20 μM danthron (A) or 100 μM anthraquinone (B), 2.5 μg/ml cytochrome P450 reductase, 250 μM $NADP^+$, 500 μM glucose-6-phosphate, 0.1 units/ml glucose-6-phosphate dehydrogenase and 500 μM $MgCl_2$ in 200 μl of 10 mM bicarbonate buffer (pH 7.8) containing 5 μM DTPA were incubated for 30 min at 37 °C. Subsequently, the ^{32}P-5'-end-labeled 98-base pair DNA fragment, 2 μM/base of calf thymus DNA and 20 μM $CuCl_2$ were added to the mixtures, followed by the incubation for 120 min at 37 °C. The DNA fragments were treated by the method described in the legend to Figure 1. The relative amounts of DNA fragments were measured by scanning the autoradiogram with a laser densitometer (LKB 2222 UltroScan XL). The horizontal axis shows the nucleotide number of the c-Ha-*ras*-1 protooncogene.

ACG sequence is complementary to the codon 273 (a known hotspot) in exon 8 of the *p53* gene.[25,26] When denatured DNA was used, preferential damage occurred more frequently at every guanine residue (Figure 4A-2). Similar DNA cleavage patterns were observed with anthraquinone (Figures 3B, 4B-1 and 4B-2).

Formation of 8-oxodG by Anthraquinones

Figure 5 shows 8-oxodG formation in calf thymus DNA treated with various concentrations of danthron and anthraquinone. The formation of 8-oxodG by these anthraquinones significantly increased at 2 μM compared with the controls ($p < 0.05$). Danthron significantly increased the formation of 8-oxodG compared with anthraquinone at 2–20 μM ($p < 0.05$). The formation of 8-oxodG by danthron decreased at 20 μM, suggesting that 8-oxodG was converted to another oxidative products.

Formation of Free Radicals from Anthraquinones

The ESR spectrum of a radical generated by cytochrome P450 reductase plus NADPH-mediated reduction of danthron is shown in Figure 6B. No signal was observed in the absence of NADPH or P450 reductase (data not shown). Figure 6A shows the computer simulation for the semiquinone radical using hyperfine splitting constants ($a^H = 0.07$ mT(6H)). This study demonstrated for the first time the hyperfine splitting constants of the semiquinone radical. Hyperfine splitting constants were estimated by reference to a report concerning semiquinone radicals.[11,12] The addition of Cu(II) resulted in a marked decrease in the signal intensity of the radical (Figure 6C). These findings suggest that Cu(II) can react with the radical. Catalase did not inhibit the formation of the radical (data not shown). Little or no signal was observed with anthraquinone in the presence of cytochrome P450 reductase and the NADPH-generating system (data not shown).

DISCUSSION

The present study has shown that carcinogenic danthron (1,8-dihydroxyanthraquinone) and anthraquinone efficiently caused Cu(II)-mediated oxidative DNA damage in the presence of cytochrome

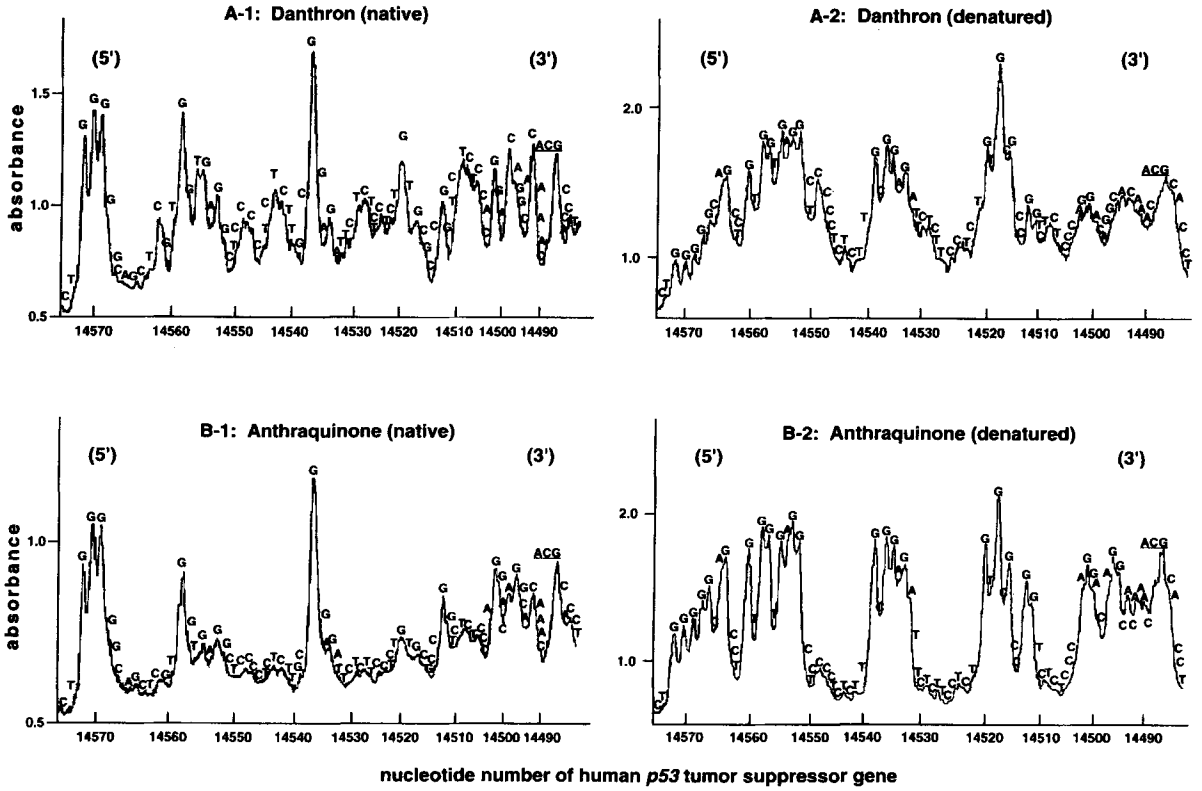

FIGURE 4 Site specificity of DNA damage by anthraquinones. The reaction mixtures containing 20 μM danthron (A-1, A-2) or 100 μM anthraquinone (B-1, B-2), 2.5 μg/ml cytochrome P450 reductase, 250 μM $NADP^+$, 500 μM glucose-6-phosphate, 0.1 units/ml glucose-6-phosphate dehydrogenase and 500 μM $MgCl_2$ in 200 μl of 10 mM bicarbonate buffer (pH 7.8) containing 5 μM DTPA were incubated for 30 min at 37 °C. Subsequently, the ^{32}P-5′-end-labeled 443-base pair DNA fragment, 2 μM/base of calf thymus DNA and 20 μM $CuCl_2$ were added to the mixtures, followed by the incubation for 120 min at 37 °C. The DNA fragments were treated by the method described in the legend to Figure 1. The relative amounts of DNA fragments were measured by scanning the autoradiogram with a laser densitometer (LKB 2222 UltroScan XL). The horizontal axis shows the nucleotide number of the human *p53* tumor suppressor gene and underscoring shows complementary sequence to codon 273 (nucleotide number 11486–11488).

P450 reductase and the NADPH-generating system. Danthron induced DNA damage more efficiently than anthraquinone. It is noteworthy that danthron and anthraquinone caused cleavage at guanine of the 5′-AC<u>G</u>-3′ sequence of the codon 273, a known hotspot[25,26] of the *p53* tumor suppressor gene. In addition, danthron and anthraquinone caused DNA damage at consecutive guanines, particularly at the 5′-<u>GG</u>-3′, 5′-<u>GGG</u>-3′ and 5′-<u>GGG</u>GG-3′ sequences in the human c-Ha-*ras*-1 protooncogene and the *p53* tumor suppressor gene. The sequence specificity of DNA damage at consecutive guanines may be explained on the basis of the highest occupied molecular orbital (HOMO) distribution. A large part of electrons of HOMO is located on the 5′-G of GG and GGG sequences,[27] and therefore, this guanine is likely to be oxidized. Sequence-selective Cu(II) binding to DNA could be explained by sequence-dependent variations of the distribution of electrons of HOMO. Thus, the sequence specificity of oxidative DNA damage may be caused by binding of copper to this guanine, lowering of ionization potential and 5′-localization of electron density of HOMO on GG bases.

ESR study confirmed that cytochrome P450 reductase plus NADPH mediated the reduction of danthron to the semiquinone radical even

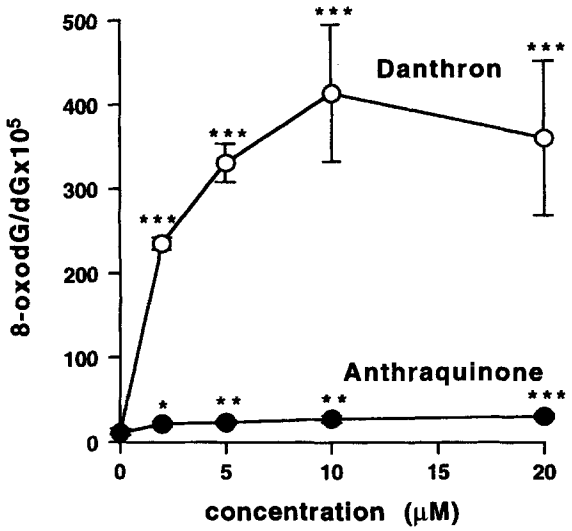

FIGURE 5 Formation of 8-oxodG by anthraquinones. The reaction mixture containing danthron (○) or anthraquinone (●), 2.5 μg/ml cytochrome P450 reductase, 100 μM $NADP^+$, 500 μM glucose-6-phosphate, 0.1 units/ml glucose-6-phosphate dehydrgenase and 500 μM $MgCl_2$ in 10 mM bicarbonate buffer (pH 7.8) containing 5 μM DTPA were incubated for 30 min at 37 °C. Subsequently, 100 μM/base calf thymus DNA and 20 μM $CuCl_2$ were added to the mixtures, followed by the incubation for 120 min at 37 °C. To stop the reaction, 0.2 mM DTPA was added and the DNA was precipitated in ethanol. The DNA fragment was enzymatically digested into nucleosides, and 8-oxodG formation was measured with an HPLC-ECD. Values represent means ± SD of three independent experiments except the control (containing neither danthron nor anthraquinone, four experiments). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, compared with the control.

in the absence of Cu(II). This result suggests that cytochrome P450 reductase and NADPH were necessary for the radical formation from danthron. Catalase completely inhibited DNA damage by danthron but did not inhibit the formation of the semiquinone radical, suggesting that this radical is not the main reactive species causing DNA damage.

In order to clarify what kinds of reactive species cause DNA damage, scavenger effects on the DNA damage and its site specificity were examined. Catalase and bathocuproine inhibited the DNA damage, suggesting that H_2O_2 reacts with Cu(I) to produce reactive species capable of causing DNA damage. Typical •OH scavengers showed no inhibitory effect on the DNA damage. Danthron induced the site-specific DNA

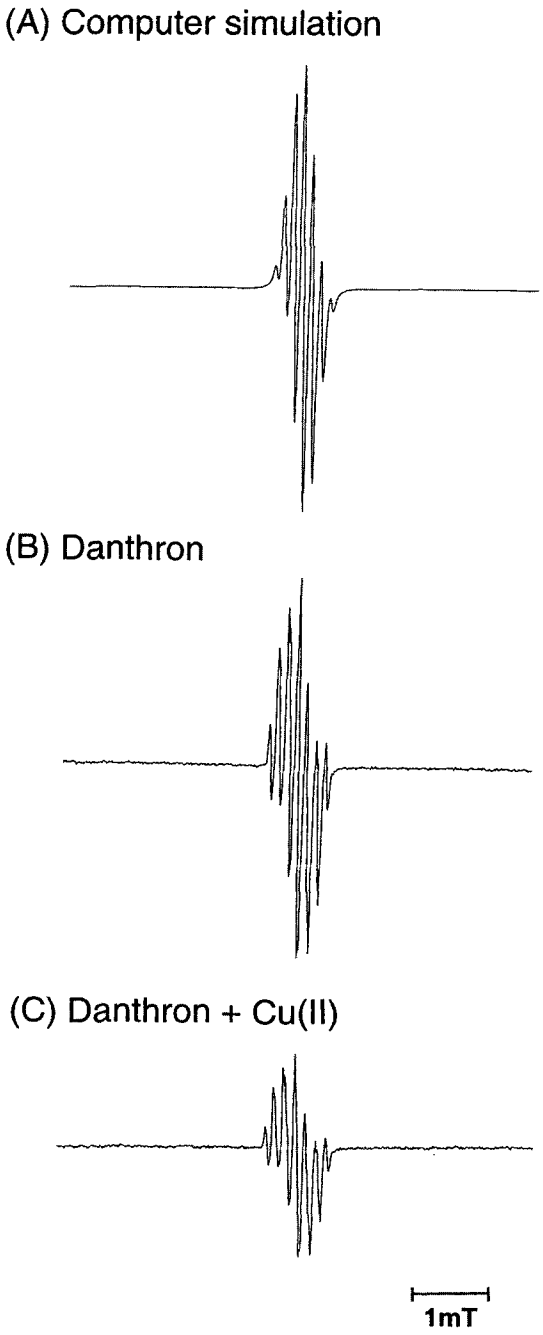

FIGURE 6 ESR spectrum of the radical generated by cytochrome P450 reductase-catalyzed reduction of danthron. The reaction mixture contained 6 mM danthron, 6 mM NADPH and 2 mg/ml cytochrome P450 reductase in 40 mM phosphate buffer (pH 7.8) containing 2 μM DTPA, and the spectrum was recorded immediately after mixing (A). Computer simulation of the semiquinone radical generated by reduction of danthron was carried out ($a^H = 0.07$ mT(6H)) (B).

damage frequently at consecutive guanine residues, while •OH causes DNA damage at every nucleotide with little site specificity.[22,28] Methional, which scavenges not only •OH but also crypto-OH radicals,[23,24] completely inhibited the DNA damage. Rodriguez *et al.*[29] reported that Cu(II)/ascorbate/H_2O_2-mediated DNA damage in aerobic aqueous solutions may be induced *in vitro* and *in vivo* through formation of a DNA-Cu(I)-H_2O_2 complex. On the basis of the present findings, and referring to several papers,[29–31] it is considered that the species causing DNA damage is a kind of crypto-OH radical, such as the Cu(I)-OOH complex. Alternatively, it is possible that •OH is formed in the immediate vicinity of DNA bases so that typical •OH scavengers cannot react with •OH before •OH reacts with DNA bases.

Some quinones can undergo enzymatic and nonenzymatic redox cycling with their corresponding semiquinone radicals and generate O_2^-.[6–13] A variety of quinone derivatives are highly redox active molecules capable of causing oxidative DNA damage through the generation of reactive oxygen species. Relevantly, Akman *et al.*[9] reported that DNA base modifications were induced in isolated human chromatin by NADH dehydrogenase-catalyzed reduction of doxorubicin, a derivative of anthraquinone. Anthraquinones appear to induce DNA damage in a similar manner, althrough these compounds are less likely to be reduced non-enzymatically and require enzymatic reduction for DNA damage. On the basis of these results, and referring to several papers,[11,12,29–31] the proposed mechanism of DNA damage induced by danthron is shown in Figure 7. Danthron is reduced by cytochrome P450 reductase and NADPH to the semiquinone radical. This radical reacts with O_2 to yield O_2^-, which is dismutated to H_2O_2. Cu(II) is reduced to Cu(I) by the interaction with O_2^-, and H_2O_2 reacts with Cu(I) to form reactive species capable of causing DNA damage. Copper is present in the mammalian cell nucleus and may contribute to high-order chromatin structures.[32] Although anthraquinone appears to induce DNA damage in a similar manner, anthraquinone induced DNA damage to a lesser extent than danthron. This finding could be explained by assuming that anthraquinone less efficiently undergo the reduction catalyzed by cytochrome P450 reductase plus NADPH than danthron. In the case of anthraquinone, its hydroxylated metabolites with similar reactivity to danthron may participate in DNA damage.

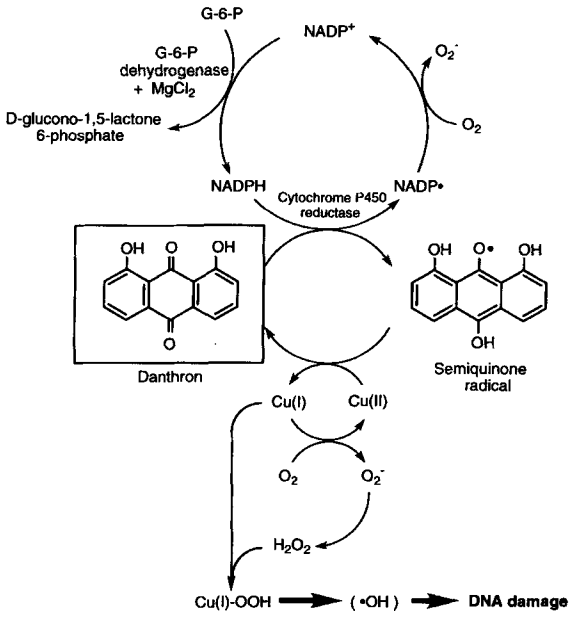

FIGURE 7 Proposed mechanism of DNA damage induced by danthron in the presence of cytochrome P450 reductase, the NADPH-generating system and Cu(II).

The present study demonstrated that danthron efficiently caused oxidative DNA damage in the presence of Cu(II), cytochrome P450 reductase and the NADPH-generating system. In particular, danthron caused DNA damage at consecutive guanines. The experiment using HPLC-ECD showed the formation of 8-oxodG at consecutive guanines, although many other oxidative products may be formed.[33,34] These findings suggest that 8-oxodG is presumably formed at these guanines, and subsequently, 8-oxodG may be further converted to piperidine-labile oxidized form of 8-oxodG, such as guani-

dinohydantoin and 2-aminoimidazolone.[35] The formation of 8-oxodG and guanidinohydantoin is known to cause G→T transversion.[36] Guanidinohydantoin and 2-aminoimidazolone may lead to G→C transversion.[37] These transversions might lead to mutation and subsequently cause cancer. We conclude that Cu(II)-mediated oxidative DNA damage by danthron and anthraquinone seems to be relevant for the expression of its carcinogenicity.

Acknowledgements

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