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Oxidative DNA Damage Induced by Pirarubicin, an Anthracycline Anticancer Agent, in the Presence of Copper(II)

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Abstract. *Background/Aim: One mechanism of the anticancer action of anthracyclines is believed to be oxidative DNA damage. Previously, we reported that doxorubicin induced oxidative DNA damage in the presence of Cu(II). However, the mechanism of pirarubicin-induced oxidative DNA damage has not been well clarified. Materials and Methods: DNA damage by pirarubicin in the presence of Cu(II) was analyzed using pBR322 plasmid DNA. $O_2^{\bullet-}$ derived from pirarubicin in the presence of Cu(II) was detected by cytochrome c reduction. Results: Pirarubicin induced DNA damage in the presence of Cu(II). Scavenger experiments suggest that reactive species are generated from H_2O_2 and Cu(I). Pirarubicin induced $O_2^{\bullet-}$ production in the presence of Cu(II). Conclusion: These findings suggest that pirarubicin plus Cu(II) induces oxidative DNA damage in a similar manner to doxorubicin, and Cu(II)-mediated oxidative DNA damage may serve as a common mechanism for antitumor effects of anthracyclines.*

Pirarubicin (4'-*O*-tetrahydropyranyl doxorubicin, THP; Figure 1A) is an anthracycline which was synthesized from doxorubicin (Figure 1B) by Umezawa *et al.* in 1979 (1). THP has a potent anticancer activity against a variety of solid cancer types as well as blood cancer (2, 3). The cytotoxicity of THP against cultured tumor cells was found to be equal or superior compared to that of doxorubicin, and the cardiotoxicity of THP was less than that of doxorubicin in hamsters (4, 5). THP is clinically approved in Japan for treatment of cancer of the head and neck, stomach, upper urinary tract, uterus and ovary, as well as acute leukemia and malignant lymphoma. Recently, it was reported that THP appeared to be less cardiotoxic than daunorubicin in the treatment of childhood acute lymphoblastic leukemia in a clinical trial (6). THP drug delivery systems developed by Maeda *et al.* have high tumor-targeting efficiency by enhanced permeability and retention effect (7, 8). *N*-(2-Hydroxypropyl)methacrylamide (HPMA) copolymer-conjugated THP was also highly effective for a patient with stage IV prostate cancer and extensive lung and bone metastases (9). Therefore, THP has recently once again gained attention in the field of cancer chemotherapy.

The anticancer action of anthracyclines is believed to be through DNA damage. DNA damage is caused by topoisomerase II inhibition, DNA intercalation and reactive oxygen species (ROS) generation (10). Recently, we reported that THP induced apoptosis through the generation of ROS (11). However, the mechanism of THP-induced DNA damage has not well been clarified. In this study, we investigated the mechanism of THP-induced DNA damage using plasmid DNA in the presence of copper (Cu) (II). In addition, we examined $O_2^{\bullet-}$ production induced by THP in the presence of Cu(II).

Materials and Methods

Materials. Pirarubicin, superoxide dismutase (SOD; 3,000 U/mg from bovine erythrocytes), catalase (45,000 U/mg from bovine liver) and cytochrome c (from equine heart) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Plasmid DNA (pBR322) and DNA gel loading dye (6×) were from Toyobo Co. (Osaka, Japan). Copper chloride ($CuCl_2 \bullet 2H_2O$) was from Nacalai Tesque Co (Kyoto, Japan). Diethylenetriamine-*N,N,N',N'',N''*-penta-

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Key Words: Pirarubicin, DNA damage, ROS, copper.

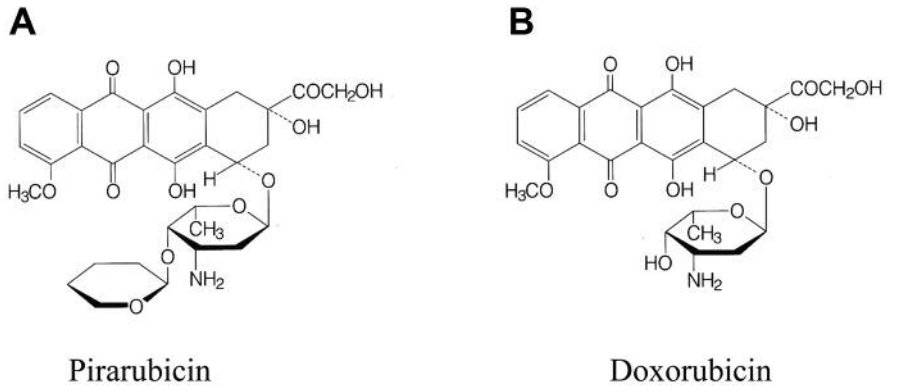

Figure 1. *Chemical structure of pirarubicin and doxorubicin.*

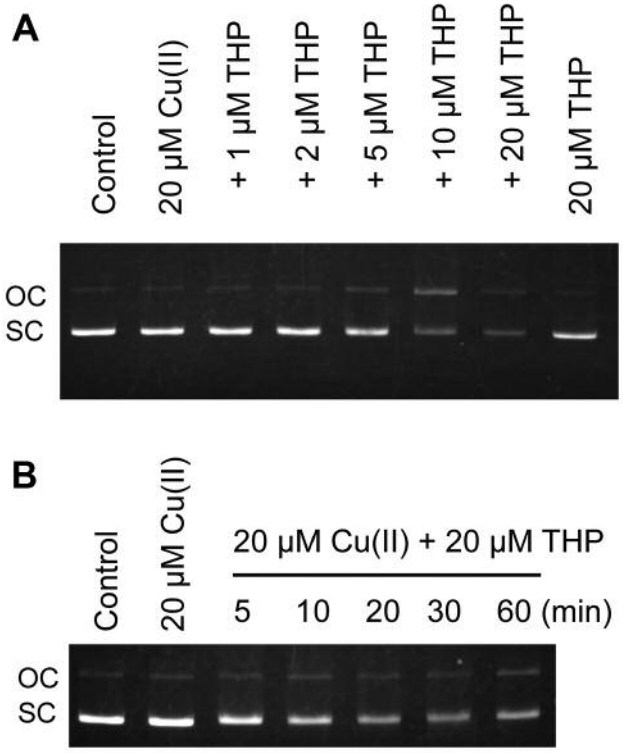

Figure 2. *Agarose gel electrophoretic patterns for the cleavage of plasmid DNA pBR322 treated with pirarubicin (THP). A: The plasmid DNA was treated with the indicated concentrations of THP in the presence of 20 μM $CuCl_2$ at 37˚C for 1 h. B: The plasmid DNA was treated with 20 μM THP in the presence of 20 μM $CuCl_2$ at 37˚C for the indicated times. The supercoiled (SC) and open circular (OC) forms of DNA are indicated.*

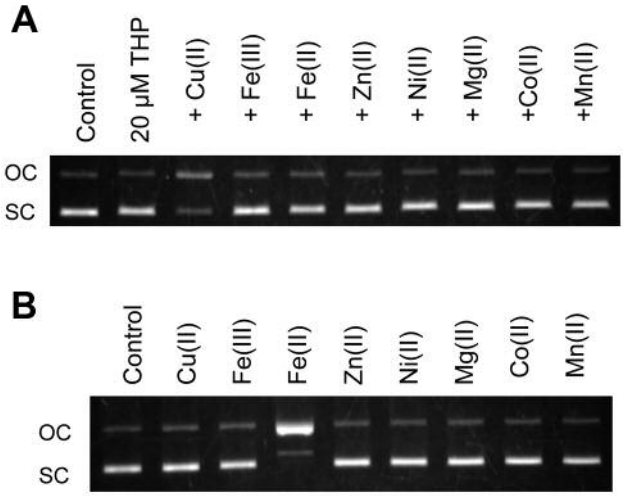

Figure 3. *Agarose gel electrophoretic patterns for the cleavage of plasmid DNA pBR322. A: The plasmid DNA were treated with 20 μM pirarubicin (THP) in the presence of 20 μM metal at 37˚C for 1 h. B: The plasmid DNA were treated with 20 μM metal at 37˚C for 1 h. Where indicated, $CuCl_2$, $FeCl_3$, $FeSO_4(NH_4)_2SO_4$, $ZnSO_4$, $NiSO_4$, $MgCl_2$, $CoCl_2$ or $MnCl_2$ was added. The supercoiled (SC) and open circular (OC) forms of DNA are indicated.*

acetic acid (DTPA) and bathocuproinedisulfonic acid were from Dojin Chemicals Co. (Kumamoto, Japan). 3-(Methylthio) propionaldehyde (methional) was from Tokyo Kasei Co. (Tokyo, Japan). All other chemicals used were of the highest purity commercially available.

Analysis of DNA damage by THP in the presence of Cu(II). The standard reaction mixture placed in a 1.5 ml Eppendorf microtube contained THP, 20 μM $CuCl_2$, pBR322 plasmid DNA (0.2 μg/tube) in 50 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA, a chelating agent, to remove trace amounts of contaminating metals. After incubation at 37˚C for 60 min, the DNA gel loading dye was added to the reaction mixture and the reacted DNA was electrophoresed on a 0.7% agarose gel containing ethidium bromide. The obtained DNA gels were analyzed by using UV transilluminator (12).

Analysis of DNA damage by THP in the presence of metals. The reaction mixture was placed in a 1.5 ml Eppendorf microtube and contained 20 μM THP, 20 μM metals [$CuCl_2$, $FeCl_3$,

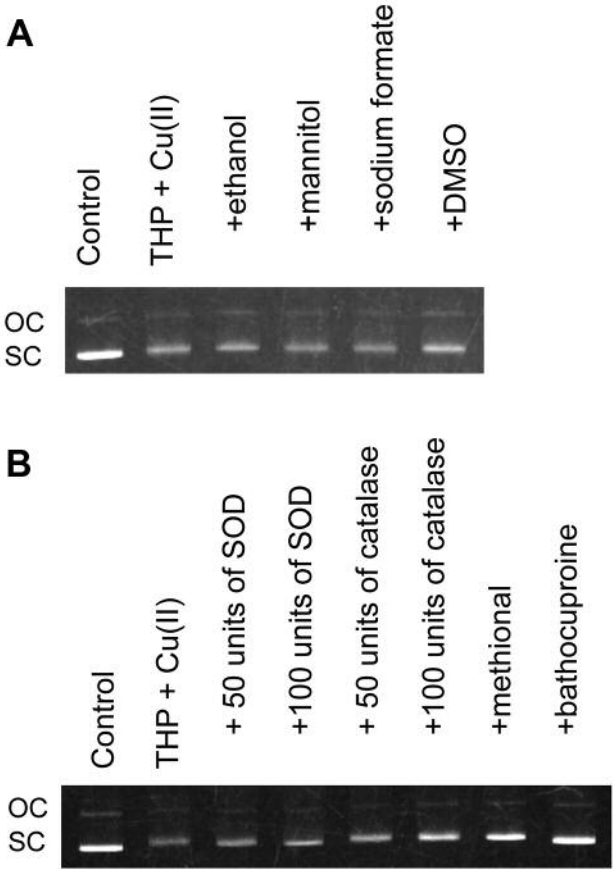

Figure 4. *Effects of reactive oxygen species (ROS) scavengers and bathocuproine on DNA cleavage induced by THP in the presence of Cu(II). The plasmid DNA was treated with 20 μM pirarubicin (THP) in the presence of Cu(II) (20 μM) at 37˚C for 1 h. Where indicated, 1.7 M (10%) ethanol, 0.1 M mannitol, 0.1 M sodium formate, 0.7 M (10%) DMSO, 50 or 100 units of superoxide dismutase (SOD), 50 or 100 units of catalase, 0.1 M methional or 50 μM bathocuproine was added. The supercoiled (SC) and open circular (OC) forms of DNA are indicated.*

$FeSO_4(NH_4)_2SO_4$, $ZnSO_4$, $NiSO_4$, $MgCl_2$, $CoCl_2$ or $MnCl_2$], and pBR322 plasmid DNA (0.2 μg/tube) in 50 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. After incubation at 37˚C for 60 min, the DNA gel loading dye was added to the reaction mixture and the reacted DNA was electrophoresed on a 0.7% agarose gel containing ethidium bromide. The obtained DNA gels were analyzed using UV transilluminator (12).

Analysis of effects of ROS scavengers and bathocuproine on DNA damage by THP in the presence of Cu(II). The reaction mixture was placed in a 1.5 ml Eppendorf microtube and contained 20 μM THP, 20 μM $CuCl_2$, and pBR322 plasmid DNA (0.2 μg/tube) in 50 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA with ROS scavengers and bathocuproine (50 μM). ROS scavengers included 1.7 M (10%) ethanol, 0.1 M mannitol, 0.1 M sodium formate, 0.7 M (10%) DMSO, 50 or 100 units of SOD, 50 or 100 units of catalase, or 0.1 M methional. After incubation at 37˚C for 60 min, the DNA gel loading dye was added to the reaction mixture and the reacted DNA was electrophoresed on a 0.7% agarose gel containing ethidium bromide. The obtained DNA gels were analyzed using UV transilluminator (12).

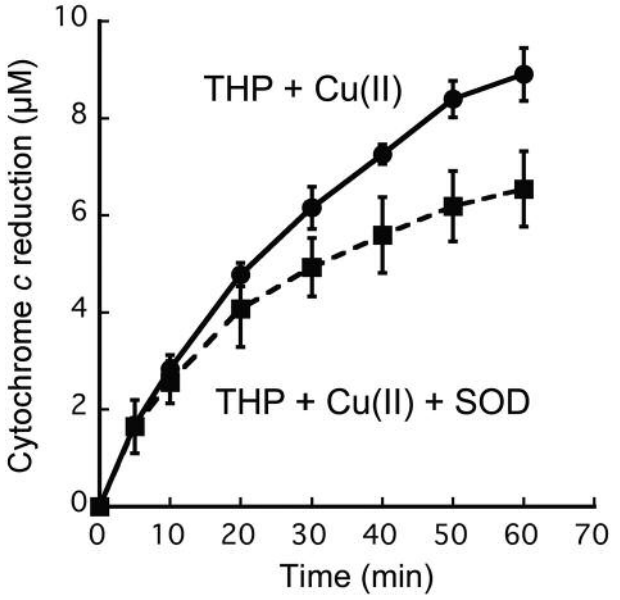

Figure 5. *Time course of cytochrome c reduction during the incubation of pirarubicin (THP) in the presence of Cu(II). The reaction mixture contained 100 μM cytochrome c, 20 μM THP in the presence 20 μM Cu(II) in 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 μM diethylenetriamine-N,N,N',N'',N''-penta-acetic acid (DTPA). A maximum absorption at 550 nm was measured at 37˚C with a UV–visible spectrophotometer. The actual amount of $O_2^{\bullet-}$ generation was calculated by subtracting absorbance with 100 U/ml superoxide dismutase (SOD) from that without SOD at 550 nm ($\varepsilon=21.1\times10^3$ M^{-1} cm^{-1}). The data were expressed as means±SD (n=3).*

Detection of $O_2^{\bullet-}$ derived from THP in the presence of Cu(II). To detect $O_2^{\bullet-}$ generation from THP, 100 μM cytochrome c was added to the reaction mixture, which contained 20 μM THP and 20 μM Cu(II) in 1 ml of 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 μM DTPA. Maximum absorption at 550 nm due to ferrocytochrome c formed by ferricytochrome c reduction was measured at 37˚C with a UV-visible spectrophotometer (UV-1700; Shimadzu, Kyoto, Japan). The actual amount of $O_2^{\bullet-}$ generation was calculated by subtracting the absorbance obtained using 100 U/ml SOD from that without SOD at 550 nm ($\varepsilon=21.1\times10^3$ M^{-1} cm^{-1}) (13).

Results

THP-induced DNA damage in the presence of Cu(II). Figure 2 shows agarose gel electrophoretic patterns for damage of plasmid DNA pBR322 treated with THP. The intensity of DNA damage increased depending on THP concentrations in the presence of Cu(II) (Figure 2A). THP induced weak DNA damage at 5 μM and apparent strong DNA damage above

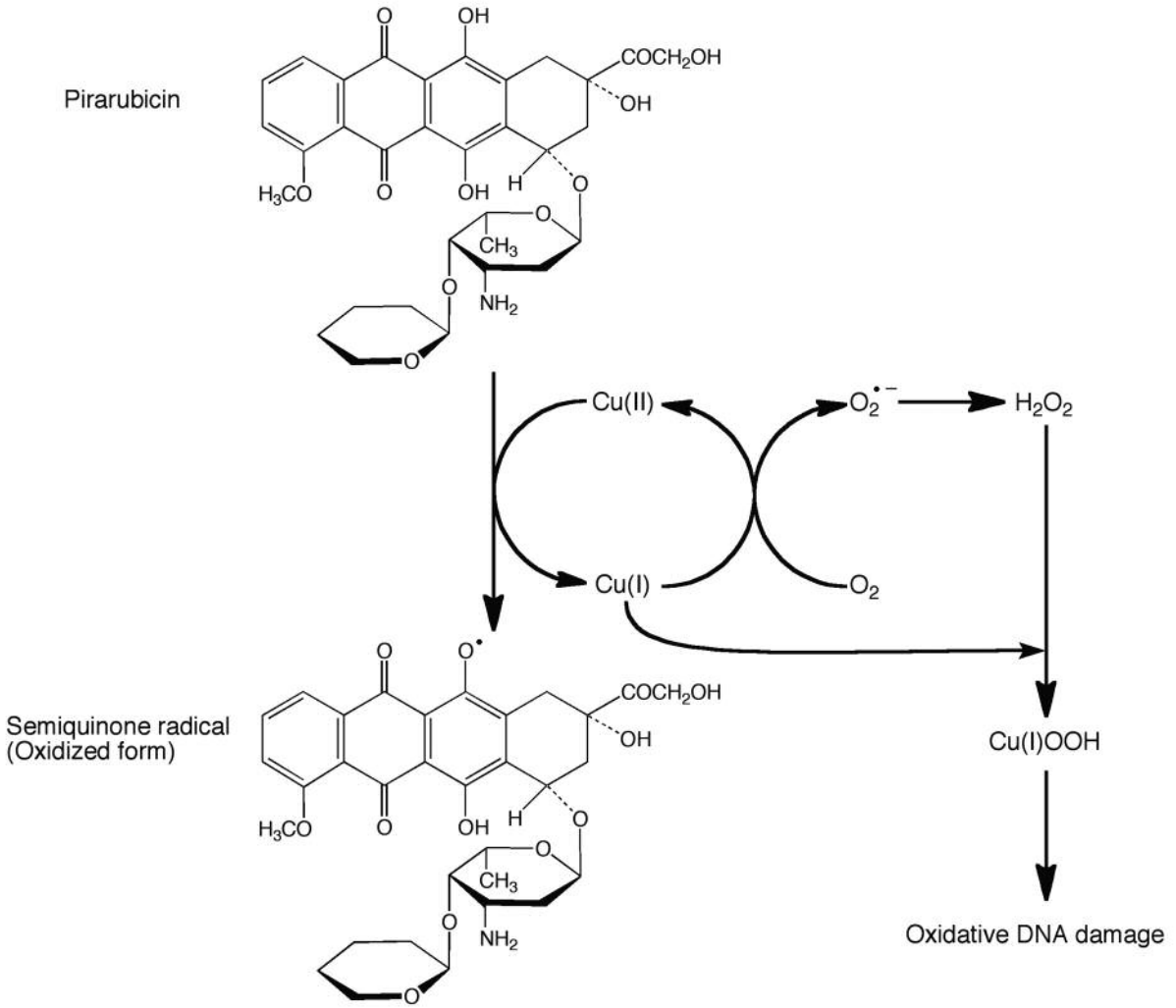

Figure 6. *Proposed mechanisms for oxidative DNA damage induced by pirarubicin in the presence of Cu(II).*

10 μM in the presence of Cu(II). Cu(II) or THP alone did not induce DNA damage. The intensity of THP-induced DNA damage increased depending on incubation time in the presence of Cu(II) (Figure 2B). THP induced apparent DNA damage above 20 min in the presence of Cu(II). On the other hand, THP did not cause DNA damage in the presence of Fe(III), Fe(II), Zn(II), Ni(II), Mg(II), Co(II) or Mn(II) (Figure 3A). However, since Fe(II) alone induced DNA damage, it is suggested that THP may chelate Fe(II) (14, 15) (Figure 3B).

Effects of ROS scavengers and bathocuproine on DNA damage by THP in the presence of Cu(II). Figure 4 shows the effects of ROS scavengers and bathocuproine on DNA damage induced by THP in the presence of Cu(II). Typical •OH scavengers, ethanol, mannitol, sodium formate and DMSO, showed little or no inhibitory effect on DNA damage (Figure 4A). SOD and catalase had inhibitory effects on DNA damage and DNA damage was completely inhibited by methional and bathocuproine (Figure 4B).

Detection of $O_2^{\bullet -}$ derived from THP in the presence of Cu(II). Figure 5 shows $O_2^{\bullet -}$ production by THP in the presence of Cu(II). The incubation of cytochome c in the presence of THP and Cu(II) led to a time-dependent increase in the cytochome c reduction. When SOD was added, the amount of cytochrome c reduction decreased, suggesting the generation of $O_2^{\bullet -}$. From these results, $O_2^{\bullet -}$ production by 20 μM THP was approximately 2.5 μM in the presence of 20 μM Cu(II). The inhibition of cytochome *c* reduction by SOD was only partial. This result suggests that there are the other $O_2^{\bullet -}$-independent mechanisms of cytochrome c reduction, probably mediated by Cu(I) (16).

Discussion

In this study, we demonstrated that THP induced oxidative damage to plasmid DNA in the presence of Cu(II) in a cell-free system. SOD and catalase had inhibitory effects on DNA damage, suggesting the involvement of $O_2^{\bullet-}$ and H_2O_2. Bathocuproine, which prevents the activation of H_2O_2 by stabilizing Cu(I) (17, 18), completely inhibited DNA damage, suggesting the involvement of Cu(I). As typical $^{\bullet}OH$ scavengers did not show inhibitory effects on DNA damage, it is suggested that free $^{\bullet}OH$ does not play an important role in DNA damage. DNA damage was completely inhibited by methional, which scavenges not only $^{\bullet}OH$ but also other radicals such as metal–oxygen complexes (19). Therefore, it is considered that ROS such as Cu(I)OOH are involved in Cu(II)-mediated DNA damage. Moreover, we demonstrated that THP generated $O_2^{\bullet-}$ in the presence of Cu(II), suggesting that THP and Cu(II) generated Cu(I), that reacted with O_2 to generate $O_2^{\bullet-}$ and subsequently H_2O_2.

We previously demonstrated that doxorubicin induced oxidative DNA damage in the presence of Cu(II) through oxidation of its p-hydroquinone moiety by copper ion (16). THP has a same aglycone moiety of doxorubicin (Figure 1). Therefore, THP appears to induce oxidative DNA damage in the presence of copper in a similar manner to doxorubicin. We propose the mechanism of THP-induced oxidative DNA damage to be as follows: THP undergoes Cu(II)-mediated one-electron oxidation at the para-OH group to generate Cu(I) and the semiquinone radical. Cu(I) reacts with O_2 to generate $O_2^{\bullet-}$ and subsequently H_2O_2. Cu(I), which is bound to DNA, interacts with H_2O_2, resulting in the formation of a DNA–copper-hydroperoxo complex [DNA-Cu(I)OOH] (Figure 6). Although typical $^{\bullet}OH$ scavengers had no inhibitory effect on DNA damage, $^{\bullet}OH$ may participate in DNA damage through the formation of DNA–Cu(I)OOH, which can release $^{\bullet}OH$ in the vicinity of DNA. $^{\bullet}OH$ immediately attacks an adjacent constituent of DNA, before it is scavenged by $^{\bullet}OH$ scavengers. In addition, our recent study demonstrated that THP induces apoptosis through the generation of H_2O_2 (11). Furthermore, it is reported that normal mouse hepatocytes were found to contain 40 μM of copper in the nucleus and 120 μM of copper in the cytosol by synchrotron X-ray fluorescent imaging (20, 21). The affinity of DNA for copper ions appears to be greater than for other essential metal ions (21, 22).

These findings suggest that THP in the presence of copper may induce oxidative DNA damage in cells. We reported that anticancer agents (doxorubicin, hydroxyurea, procarbazine, cyclophosphamide and dacarbazine) induced oxidative DNA damage in the presence of copper in a cell-free system (16, 23-26). Several previous reports stated that anthracyclines induced ROS generation and oxidative DNA damage in the presence of iron (15, 27, 28), whereas we demonstrated that copper also plays an important role in DNA-damaging activity of anthracyclines. Therefore, Cu(II)-mediated oxidative DNA damage may be a common mechanism for the antitumor effects of anthracyclines.

Acknowledgements

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