Chem. **1989**, *54*, 5110; (b) Hauser, F. M.; Rhee, R. P. *J. Org. Chem.* **1978**, *43*, 178; (c) Rho, Y. S.; Cho, I. H.; Park, S. H.; Ahn, K. H. *J. Kor. Chem. Soc.* **1993**, *37*, 141.
6. Compound 3 was obtained by subjecting diethyl adipate (100 g, 49.89 mmol) and Na (5.05 g, 219.5 mmol) to the acyloin condensation process in benzene solvent, to obtain 2-hydroxycyclohexanone (3.83 g, 67%); and by methylation with dimethylsulfate and K_2CO_3. Allingger, N. L. *Organic Syntheses*; Rabjohn, N. ed.; John Wiley & Sons: Inc; **1963**; Coll. Vol. IV. p 840.
7. (a) Reich, H. J.; Renga, J. M.; Reich, I. L. *J. Am. Chem. Soc.* **1975**, *97*, 4534; (b) Ryu, I.; Murai, S.; Hatayama, Y.; Sonoda, N. *Tetrahedron Lett.* **1978**, 3455
8. Rubottom, G. M.; Gruber, J. M. *J. Org. Chem.* **1978**, *43*, 1599.
9. (a) Trost, B. M.; Salzmann, T. N.; Hiroi, K. *J. Am. Chem. Soc.* **1976**, *98*, 4887; (b) Trost, B. M.; Salzmann, T. N. *J. Am. Chem. Soc.* **1973**, *95*, 6840; (c) Trost, B. M.; Massiot, G. S. *J. Am. Chem. Soc.* **1977**, *99*, 4405.
10. Shine, H. J.; Rahman, M.; Seeger, H.; Wu, G. S. *J. Org. Chem.* **1967**, *32*, 1901.
11. (a) Davis, B. R.; Halsall, T. G. *J. Chem. Soc.* **1962**, 1883; (b) Ishizaki, M.; Hoshino, O.; Iitaka, Y. *J. Org. Chem.* **1992**, *57*, 7285; (c) Burn, D.; Petrow, V. *J. Chem. Soc.* **1962**, 1223.
12. (a) Stotter, P. L.; Hill, K. A. *J. Org. Chem.* **1973**, *38*, 2576; (b) Garbisch, Jr. E. W. *J. Org. Chem.* **1965**, *30*, 2109.
13. Fleming, I.; Paterson, I. *Synthesis* **1979**, 736.
14. (a) Hauser, F. M.; Prasanna, S. *Tetrahedron* **1984**, *40*, 4711; (b) Grieco, P. A.; Reap, J. P. *Tetrahedron Lett.* **1974**, *36*, 1097.
15. Williard, P. G.; Fryhle, C. B. *Tetrahedron Lett.* **1980**, *21*, 3731.
16. Clark, J. H.; Holland, H. L.; Miller, J. M. *Tetrahedron Lett.* **1976**, *38*, 3361.

Cu^{2+}-Anthraquinone Complexes: Formation, Interaction with DNA, and Biological Activity

Thong-Sung Ko*, Hack-Young Maeng, Mi-Kyeong Park, Il-Hyun Park, In-Sang Park, and Byoung-Sun Kim

Department of Biochemistry, Chungnam National University, Taejon 305-764
Received December 7, 1993

Growth inhibition potency of the anthraquinones, anthraquinone-1,5-disulfonic acid and carminic acid, for Sarcoma 180 and L1210 leukemia cells *in vivo* and *in vitro*, was induced by the divalent transition metal ion, Cu^{2+}. On the other hand spectroscopic titration data show that the anthraquinone drugs form Cu^{2+} chelate complexes (carminic acid : Cu^{2+} = 1 : 6; anthraquinone-1,5-disulfonic acid : Cu^{2+} = 1 : 3). Furthermore the Cu^{2+}-drug complexes associate with DNA to form the Cu^{2+}-anthraquinone-DNA ternary complexes. The formation of the complexes was further supported by the H_2O_2-dependent DNA degradation, which can be inhibited by ethidium bromide, caused by the Cu^{2+}-drug complexes. It is likely that the Cu^{2+}-mediated cytotoxicity of the anthraquinone drugs is related with the Cu^{2+}-mediated binding of the anthraquinone drugs to DNA and DNA degradation.

Introduction

Although the anthracyline antibiotics are believed to exert their antitumor action[1] by inhibiting DNA transcription and replication *via* intercalation of the anthracycline chromophore between DNA base pairs[2], their mechanism of action remains uncertain. The planar anthracycline chromophore, which can intercalate together with the positively charged amino-sugar moiety can be anticipated to provide a high DNA affinity. As is often the case with other intercalating agents[3,4], the DNA-topoisomerase II complex is presently believed to be the most probable target of the antitumor activity of the anthracyclines, resulting in DNA strand breakage. If the potent genotoxicity and antitumor activity of adriamycin (doxorubicin) could be attributable to its possession of planar aromatic chromophore which can intercalate between DNA base pairs, such anthraquinones as anthraquinone-1,5-disulfonic acid and carminic acid should also be expected to be able to be induced to attain DNA-intercalative form and consequently the anthracycline-like activity through the formation of appropriate positively charged metal chelate complex of the drugs, although their free drug forms are negatively charged and can not bind to DNA directly due to highly unfavorable electrostatic repulsive forces between the drug and the DNA molecules of the similar charge.

The idea described above prompted us to carry out a series of spectroscopic studies on the formation of the Cu^{2+}-drug complex, and interaction of the Cu^{2+}-drug complexes with DNA to form the ternary complexes. Cu^{2+}-drug-DNA; and consequent cytotoxicity of the Cu^{2+}-drug complexes against L1210 leukemia cells in culture and antitumor activity against Sarcoma 180 *in vivo*. Here we present some of the results.

Experimental

Spectrophotometric Methods in the Test of the Formation of Metallocomplexes and the Interaction of the Complexes with DNA. The formation of Cu^{2+}-chelate complexes of the anthraquinone drugs (*i.e.*, anthraquinone-1,5-disulfonic acid and carminic acid respectively) was investigated by spectrophotometric titration in 0.05 M HEPES pH 7.4. Anthraquinone-1,5-disulfonic acid and carminic acid (Aldrich) were recrystallized once in the mixture of ethanol and chloroform prior to use, and the stock solutions were prepared just before use. Calf thymus DNA (type 1: sodium, salt, highly polymerized) was purchased from Sigma Chemical Company. DNA concentrations are stated in terms of nucleotide phosphorus by using the extinction coefficient $\varepsilon = 6600\ M^{-1}cm^{-1}$. The difference absorption spectrophotometric titration of anthraquinone-1,5-disulfonic acid with Cu^{2+} was performed at 25℃, using a double compartment cuvette, as the reference cuvette, containing the drug stock solution (twice the concentration of the reaction cuvette, *i.e.*, 2×10^{-5} M) in one compartment and the HEPES buffer in another compartment. Both the stock drug solutions and the Cu^{2+} ($CuCl_2$) solutions were all prepared in the 0.05 M HEPES buffer, pH 7.5. For the titration, successive aliquots of concentrated Cu^{2+} solution were added to both the reaction cuvette and the buffer compartment of the reference cuvette simultaneously. The stock drug concentration in the reaction cuvette was kept constant (at 1×10^{-5} M) by simultaneous addition of the same volume of drug at twice stock drug concentration (*i.e.*, 2×10^{-5} M). The titration spectra were obtained with a UVICON 860 UV-VIS spectrophotometer equipped with a NESLAB RTE 210 thermoregulator maintaining the temperature at 25℃. The titration of carminic acid with Cu^{2+} was performed with a 1 cm cuvette by the addition of successive aliquots of concentrated $CuCl_2$ to a dilute stock solution of carminic acid. The concentration of the stock carminic acid was kept constant by simulataneous addition of the same volume of drug at twice the stock drug concentration. The titration of the metal-drug complex with DNA were also performed, in the same manner as in the titration of drugs with Cu^{2+}, by the addition of successive aliquots of concentrated DNA to a dilute stock metal-drug concentration. From the plot of the normalized peak heights (fractional absorbance change) of titration spectra against the concentration of Cu^{2+} (in the case of Cu^{2+}-drug complex formation) and DNA (in the case of metal-anthraquinone-DNA ternary complex formation) respectively, the stoichiometry of the complexes in each case (of the metal-drug complex and metal-drug-DNA complex respectively) was estimated.

***In Vivo* Antitumor Test.** *Sarcoma 180* provided by the Korea Ginseng and Tobacco Research Institute was used for *in vivo* antitumor test in accordance with the protocols described by the National Cancer Institute[5]. *Sarcoma 180* cells were grown in the peritoneal cavity of male ICR mouse (19-21 g) and maintained by sequential transplantations weekly. In the experiments male ICR mouse (19-21 g) were implanted with 10^6 Sarcoma 180 cells in the peritoneal cavity on day 0 (eight mice per group). Test substances in physiological saline solution were injected in the peritoneal cavity on days 1-7 (5 ml/kg). The response was measured in median survival time (in days). Results are expressed by T/C×100 (T = median survival time of treated animals; C = median survival time of control animals). The criteria for significant activity is T/C×100>120.

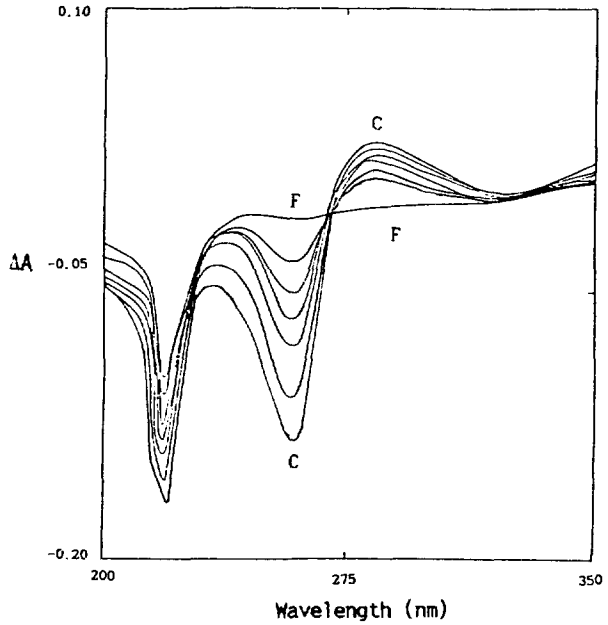

Figure 1. UV-VIS difference absorption spectra of anthraquinone-1,5-disulfonic acid in the absence and in the presence of various amounts of Cu^{2+}. The formation of Cu^{2+}-anthraquinone-1,5-disulfonic acid complex is shown by the absorption difference spectral shifts in the titration of anthraquinone-1,5-disulfonic acid at constant concentration of 1×10^{-5} M in 0.05 M HEPES buffer pH 7.5 with Cu^{2+} at various concentrations ranging from zero (top curve, F, at 260 nm) to 3×10^{-5} M (bottom curve, C, at 260 nm).

***In Vitro* Inhibition of L1210 Leukemia Cell Growth.** The cell line of mouse leukemic lymphoblast, L1210 was grown at 37℃ under 5% CO_2 in screw-capped tubes (10×160 mm) of Ficher's medium supplemented with 10% horse serum, 1.25 g/l sodium bicarbonate, 10^5 units/l penicillin, and 0.1 g/l streptomycin. The value of ED_{50}, median effective dose, was determined following the procedures described by Thayer *et al.*[6], with minor modifications to express the drug effect on growth of L1210 cells.

Test of DNA strand Cleavage Activity of the Cu^{2+}-anthraquinone Complexes. Formation of acid-soluble counts from ^{14}C-DNA (calf thymus) by Cu^{2+}-anthraquinone-1,5-disulfonic acid complexes in the presence of ascorbic acid and H_2O_2 was tested following the procedures described previously[7]. To prove the intercalative interaction of the metal complexes of the anthraquinones with the double-stranded DNA, the inhibitory effect of the typical intercalator, ethidium bromide (EtBr), on the cleavage assay was also tested in accordance with the procedure described previously[7].

Results and Discussion

The formation of Cu^{2+}-complexes of anthraquinone-1,5-disulfonic acid and carminic acid respectively was proved by spectrophotometric titrations as shown in Figure 1 and Fig-

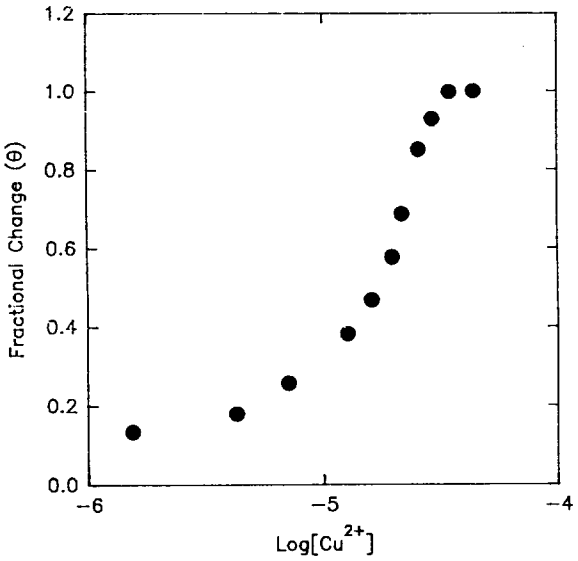

Figure 2. Plot of fractional change (θ) in absorbance at 260 nm *vs.* Log[Cu^{2+}] based on the titration data of Figure 1.

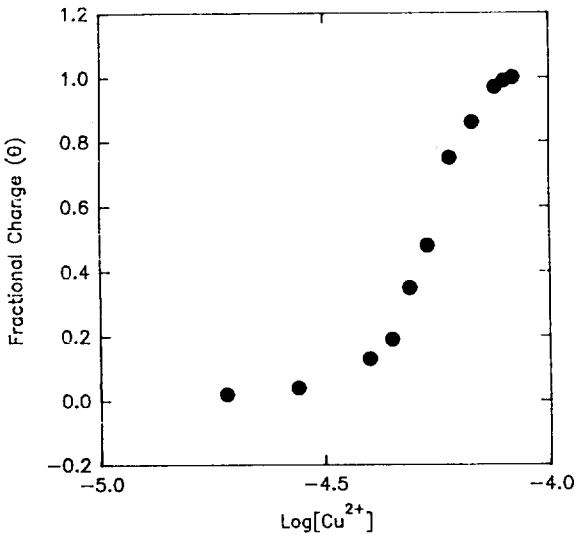

Figure 4. Plot of fractional charge (θ) in absorbance at 520 nm *vs.* Log[Cu^{2+}] based on the titration data of Figure 3.

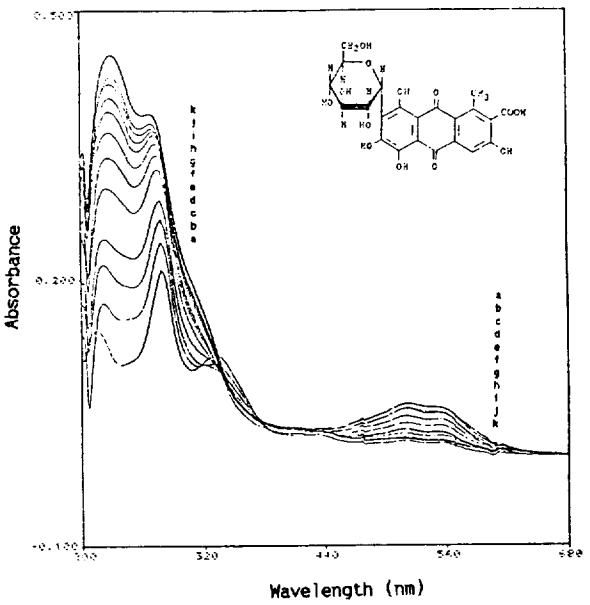

Figure 3. Absorption spectra of carminic acid in various concentrations of Cu^{2+}. Carminic acid at constant concentration of 1×10^{-5} M was titrated with Cu^{2+} of varying concentrations; (a) zero M, (b) 2.1×10^{-5} M, (c) $2.9\times10^{-}$ M, (d) 4.4×10^{-5} M, (e) 5.2×10^{-5} M, (f) 5.7×10^{-5} M, (g) 6.2×10^{-5} M, (h) 6.5×10^{-5} M, (i) 6.8×10^{-5} M, (j) 7.1×10^{-5} M, (k) 7.4×10^{-5} M. Inset shows the structure of carminic acid.

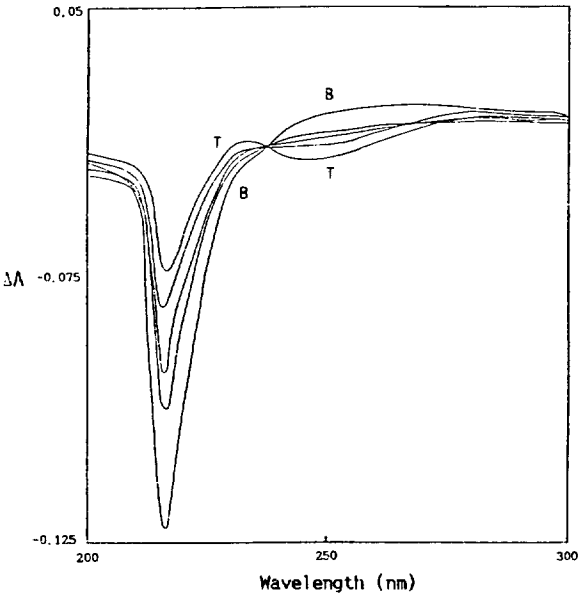

Figure 5. UV-VIS difference absorption spectroscopic demonstration of the formation of the ternary complex of Cu^{2+}-anthraquinone-1,5-disulfonic acid-DNA. The absorption difference spectra are those resulted from the titration of the mixture of 1×10^{-5} M anthraquinone-1,5-disulfonic acid and 1×10^{-5} M Cu^{2+} with calf thymus DNA in 0.05 M HEPES buffer pH 7.5 containing 0.1 M NaCl. The curve marked B is the spectrum of Cu^{2+}-anthraquinone-1,5-disulfonic acid (binary complex). The curve marked T denotes the spectrum of the titration with the highest concentration of DNA (1.5×10^{-5} M) (ternary complex), other curves represent successive decrements in DNA concentration.

ure 3. Direct binding of the anthraquinones to the polyanionic DNA is improbable in consideration of the similar negative charge on the drug molecules. However, through the formation of the drug-metal complex, the drugs will be able to bind to the DNA as in the cases of other negatively charged drugs such as streptonigrin[8] and quinolones[9]. Figure 1 shows the absorption difference-spectral shifts in the titration of anthraquinone-1,5-disulfonic acid with Cu^{2+}. Addition of Cu^{2+} causes depression of 260 nm peak of the drug and concomitant elevation of 280 nm peak with an unwell-defined isosbestic point at 275 nm, indicating the drug-metal complex formation, up to the point where the [drug]/[Cu^{2+}] ratio of 1/3 is reached (see also Figure 2). The spectrophotometric titration data for carminic acid are presented in Figure 3. Carminic acid in the HEPES buffer, pH 7.5 has an absorption

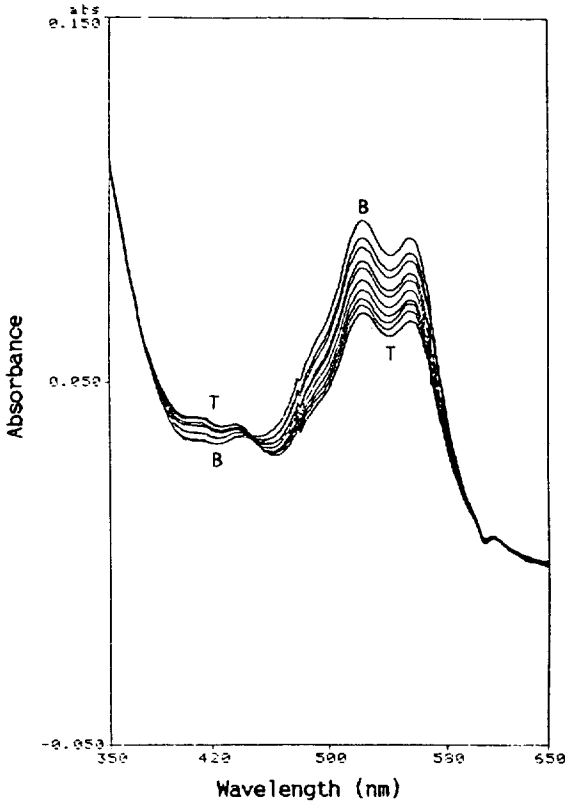

Figure 6. UV-VIS absorption spectroscopic demonstration of a ternary complex between carminic acid, Cu^{2+}, and DNA. Absorption spectra of carminic acid-Cu^{2+} (1 : 6) complex in various concentrations of DNA. Carminic acid-Cu^{2+} (1 : 6) complex at constant concentration of 1×10^{-5} M was titrated with DNA of varying concentrations. The curve marked B is the spectrum of Cu^{2+}-carminic acid (binary complex). The curve marked T denotes the spectrum of the titration with the highest concentration of DNA (ternary complex). The DNA concentrations from the highest absorbance maximum to the lowest, respectively, were; 0.0, 0.39, 0.75, 1.1, 1.4, 1.7, 2.0, 2.5, 2.8, 3.0, 3.4, 3.8, 4.8 ($\times10^{-5}$ M).

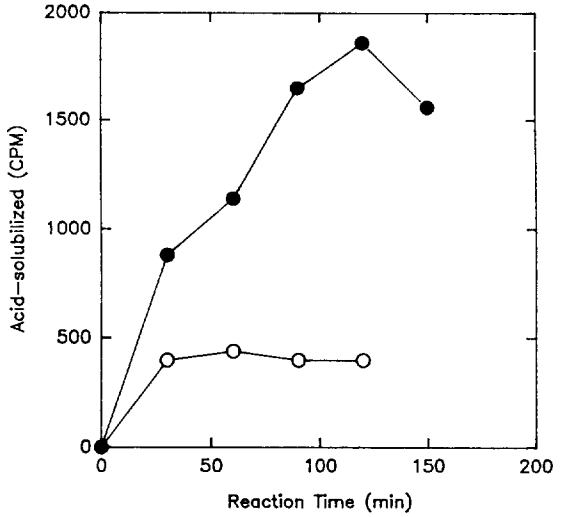

Figure 7. H_2O_2-dependent DNA degradation induced by Cu^{2+}-anthraquinone-1,5-disulfonic acid complexes. Formation of acid-soluble counts from ^{14}C-DNA by anthraquinone-1,5-disulfonic acid-Cu^{2+} (5×10^{-5} M) in the presence of ascorbic acid (10 mM) and H_2O_2 (10 mM) were assayed. The assay was carried out in a total volume of 0.6 m*l* of 0.05 M HEPES buffer pH 6.5 at 35℃. (●—●); in the presence of anthraquinone-1,5-disulfonic acid-Cu^{2+} complex, (○—○); in the presence of Cu^{2+} alone.

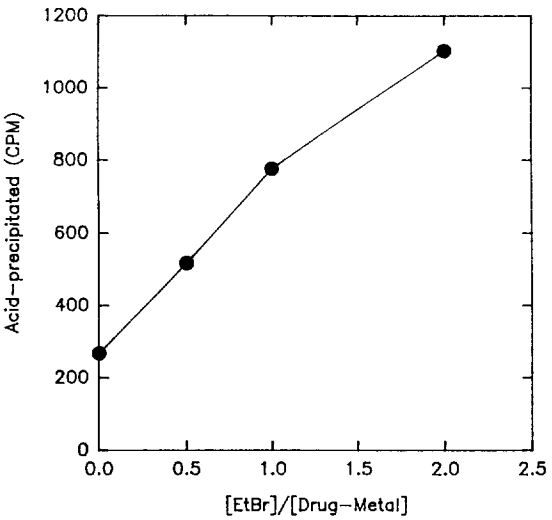

Figure 8. Inhibitory effect of intercalator ethidium bromide (EtBr) on the formation of acid-soluble counts from ^{14}C-DNA by anthraquinone-1,5-disulfonic acid-Cu^{2+} (5×10^{-5} M).

maximum at 520 nm. Addition of Cu^{2+} causes depression of this maximum up to the point the [drug]/[Cu^{2+}] ratio of 1/6 is reached (see also Figure 4). Figure 2 and Figure 4 show the variations of the normalized absorbances of the titration spectra of the anthraquinone drugs (anthraquinone-1,5-disulfonic acid and carminic acid respectively) with the concentration of the Cu^{2+} added. From the plot of fractional change (θ) *vs.* log[Cu^{2+}] in Figure 2 constructed from the titration data of Figure 1 for anthraquinone-1,5-disulfonic acid (at the constant concentration of 1×10^{-5} M), the stoichiometry for the metallodrug complex can be estimated to be anthraquinone : Cu^{2+} = 1 : 3. In the same way, the stoichiometry of carminic acid : Cu^{2+} = 1 : 6 was obtained from the data of Figure 4. In the tests of the cytotoxicity and antitumor activity of these metallodrugs these values of stoichiometry were used in the construction of the Cu^{2+}-drug complexes. Spectrophotometric evidences for the association of the metallodrugs with DNA to form the ternary complexes of drug-Cu^{2+}-DNA were obtained from the difference absorption spectroscopic titration data in Figure 5 for the formation of ternary complex, Cu^{2+}-anthraquinone-1,5-disulfonic acid-DNA complex and the absorption spectroscopic titration data in Figure 6 for the formation of Cu^{2+}-carmic acid-DNA complex. In Figure 5, addition of DNA causes suppression of the difference spectra around 250 nm (curve B) and blue-shift of the maximum (curve T), indicating complex formation. Intermediate concentrations of DNA reveal the existence of a fairly clear-cut isosbestic point at 236 nm. The data for the absorption spectroscopic titration of carminic acid-Cu^{2+} (1 : 6) complex with DNA are shown in Figure 6. Addition of DNA causes suppression of the absorption maximum at 520 nm (curve B) and blue shift of the maximum (curve

Table 1. Effects of anthraquinone-Cu(II) complex formation on *in vivo* and *in vitro* cytotoxicity

Drug species	Ratio of Drug : Cu^{2+}	ED_{50}(μg/ml) (for L1210)	T/C×100 (for Sarcoma 180)
Anthraquinone-1,5-disulfonic acid	1 : 0	No cytotoxicity	94.4
Anthraquinone-1,5-disulfonic acid-Cu^{2+}	1 : 3	1.904	122.4
Carminic acid	1 : 0	No cytotoxicity	111.1
Carminic acid-Cu^{2+}	1 : 6	1.23	118.7
Cu^{2+} alone		No cytotoxicity	–

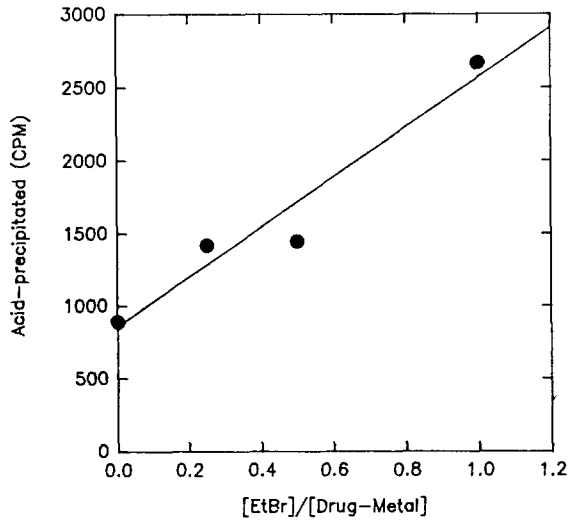

Figure 9. Inhibitory effect of intercalator ethidium bromide (EtBr) on the formation of acid-soluble counts from ^{14}C-DNA by carminic acid-Cu^{2+} (1 : 6) complex.

T), indicating complex formation. Intermediate concentrations of DNA reveal the existence of an isosbestic point at 451 nm. There could be two ways in which Cu^{2+} ions induce the formation of the ternary complexes (anthraquinone-Cu^{2+}-DNA): *via* changing the net charge on the anthraquinone molecules from negative to positive, thereby changing the ionic interaction between the drug and the DNA phosphates from repulsive to attractive; *via* increasing the planarity of the aromatic chromophore configurations by the formation of metal-chelate rings (analogous to the cases of quinolones[9]). In order to get affirmative evidence for the formation of the ternary complexes and to test intercalative nature of the ternary complexes, we performed the tests for H_2O_2-dependent DNA cleavage reaction catalyzed by the anthraquinone-Cu^{2+} complexes, and for the inhibitory effect of typical intercalator ethidium bromide (EtBr) on the cleavage. The results shown in the figures, Figure 7 to Figure 9, support the spectroscopic evidences for the formation of the ternary complex (anthraquinone-Cu^{2+}-DNA). The hydroxyl radical produced *in situ* by the DNA-associated anthraquinone-Cu^{2+} complex may act on and cleave the DNA strand, whereas the hydroxyl radical produced by the anthraquinone-Cu^{2+} complex free and separated from the DNA strand may be decayed before it reaches the DNA site for cleavage. Figure 7 demonstrates the H_2O_2-dependent DNA cleavage reaction catalyzed by (anthraquinone-1,5-disulfonic acid)-Cu^{2+} complex, indicating the association of the metal-drug complex with the DNA. Inhibitory effect of the typical intercalating agent, ethidium bromide, on these degradation of DNA by the Cu^{2+}-complexes of anthraquinone-1,5-disulfonic acid and carminic acid respectively, suggest the possibility that the anthraquinone-Cu^{2+} complexes intercalate into the DNA. Further more works will be required to verify this possibility and a possible role for intercalative intercaction in these ternary complexes remains to be investigated.

Induction of growth inhibition potency of the anthraquinones (anthraquinone-1,5-disulfonic acid and carminic acid) against L1210 leukemia cells in culture and Sarcoma 180 *in vivo* respectively by Cu^{2+} is shown in Table 1. In the table the *in vitro* inhibition of L1210 leukemia cell growth and *in vivo* inhibitioin of Sarcoma 180 cell growth respectively by the Cu^{2+}-anthraquinone complex species are compared with those exerted by the free drugs. The data summarized in the table show *in vitro* and *in vivo* induction of cytotoxicity (antitumor activity) of anthraquinone-1,5-disulfonic acid and carminic acid, which are both anionic in their free drug froms, by Cu^{2+}. The metals, Cu^{2+} ions, themselves alone did not show significant cytotoxic effects under the conditions tested.

One may imagine that other transitioin metal divalent cations such as Mn^{2+}, Cd^{2+}, Zn^{2+}, and Ni^{2+} may show similar effects. This paper presents evidence that under appropriate conditions the anthraquinones will bind to DNA and cause lethal effect on certain cells. It is likely that their effect of association with DNA is linked with their cytotoxicity. The elucidation of this relationship awaits further extensive research works.

Acknowledgement. This work was supported by the Research Fund 1988, Ministry of Education, Korea.

References

1. Crooke, S. J.; Reich, S. D. In *Anthracyclines Current Status and New Developments;* Academic Press: New York, 1980.
2. Arcamone, F. In *Topics in Antibiotic Chemistry;* Sammes, G. P. Ed.; Wiley: New York, 1978, 2, p 102-129.
3. Drlica, K.; Franco, R. *Biochemistry* **1988**, *27*, 2253.
4. Ross, W. E. *Biochem. Pharmacol.* **1985**, *34*, 4191.
5. Geran, R. I.; Greenberg, N. H.; MacDonald, M. M.; Schumacher, A. M.; Abott, B. J. *Cancer Chemother. Rep.* **1972**, *Part 3*, 1-103.
6. Thayer, P. S.; Himmelfarb, P.; Watts, G. L. *Cancer Chemother. Rep.* **1971**, *Part 2*, 1.
7. Ko, T.-S.; Park, M. -K.; Ryu, H.-W.; Suh, I.-H. *Korean Biochem. J.* **1990**, *23*, 465.
8. White, J. R. *Biochem. Biophy. Res. Comm.* **1977**, *77*, 387.
9. Ko, T.-S.; Kwon, T.-I.; Kim, M.-J.; Park, I.-H.; Ryu, H.-W. *Bull. Korean Chem. Soc.* **1994**, *15*.