



Experimental Antitumor Activity of the Ce(IV)-Mitoxantrone Complex and Its Interaction with Deoxyribonucleic Acid

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Abstract

The Ce(IV)-mitoxantrone complex exhibits a higher lethality to Ehrlich ascites tumor cells than that of the free drug and shows stronger inhibition ability on the DNA synthesis of the tumor cells. Thus the Ce(IV)-mitoxantrone complex may become a more potent antitumor drug than mitoxantrone. The different interaction model of mitoxantrone and its Ce(IV) complex with DNA were studied by the methods of spectroscopy, electrochemistry, and electrophoresis. Ce(IV) ions chelate with oxygens of the hydroxyl groups at the 1,4 position and the carbonyl function on C-9 and C-10, then intercalate into the base pairs of DNA together. The complexation of Ce(IV) gives rise to more compact binding of mitoxantrone with DNA, and leads to an additional change on the normal conformation and the double-helical structure of DNA; this may be related to the more stronger action of the complex on DNA synthesis and survival of cultured tumor cells. Journal of Inorganic Biochemistry 68, 117–121 (1997) © 1997 Elsevier Science Inc.

Introduction

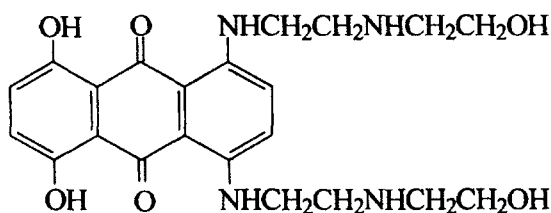
Anthracenedione mitoxantrone (1,4-dihydroxy-5,8-bis [[2- [(2-hydroxyethyl)-amino] -ethyl]amino]-9, 10-anthracene-dione dihydrochloride, MX), a new synthetic analogue of the anthracycline antibiotics, has shown significant clinical effectiveness in the treatment of breast cancer, lymphoma, and acute leukemia [1]. In contrast to other anthracycline antitumor drugs, mitoxantrone produces less side effects such as cardiac toxicity [2]. The structure of mitoxantrone is shown in Scheme I. Studies of cell biology and biochemistry indicated that nucleic acids are the main cell target of mitoxantrone, it binds to DNA with high affinity, at least in part by intercalation [3], but via electrostatic interactions as well [4]. Mitoxantrone was shown to induce compaction of isolated chromatin [5], as well as protein-associated DNA cleavage [6], and to inhibit macromolecular biosynthesis in a number of tumor cell lines [7]. The most dominant molecular mechanism of antitumor action of mitoxantrone appears to be the induction of long-term DNA damage [8].

The interaction of the Pd(II)-MX complex with DNA was studied by circular dichroism spectra [9]. The Pd(II) ion is bound to the N atoms of the side chains of mitoxantrone, and the complex exhibited antitumor ability similar to that of the free drug. The complexation of Pd(II) ion prevents the intercalation of the drug between the base pairs of DNA, while having the same tendency to aggregate and precipitate nucleic acids. We studied the antitumor activity of the Cu(II)-MX complex and its interaction with DNA [10]. Similar to Pd(II) ions, the Cu(II) complex with the nitrogens of the side chains of mitoxantrone; and the binding strength of intercalation is weakened due to the complexation of Cu(II) ions, but it increases the ability of MX to change the conformation of DNA and to cause its denaturation. Both Cu(II) and Pd(II) are soft metallic ions having preference affinity to nitrogen atoms. Thus we then select the hard acids ions Fe(III) and Ce(IV), which having preference affinity to oxygen atoms, to complex with MX. We find that the Fe(III)-MX complex shows no increasing of experimental antitumor activity than that of MX, while the Ce(IV)-MX complex shows a stronger action on the survival of cultured tumor cells than that of MX. We conclude that the complexation of the different metals with MX leads to the different acting model, i.e., the Ce(IV)-MX complex with DNA is different from that of Cu(II) and Pd(II) complexes.

Rare earth elements have obviously biological effects such as antibacterial, antiinfective, and anticoagulant action. The lanthanide isotopes were used in therapy and diagnosis of the tumors [11], there are also the reports that the compounds of lanthanum have antitumor activity [12], such as certain sulphate, lanthanum chloride, and lanthanum glycinate. Cerium is a rare earth element, there were several compounds of cerium used in the clinical approach, such as Ce(NO₃)₃ was used in the treatment of superficial burns in man; Ce(SO₄)₂, as an antibacterial drug, in the treatment of wounds. So this complexation may display synergism in the antitumor action. In addition to this, the complexation of Ce(IV) is equated to the structure modification of the drug; by analyzing the different action models between them, will be helpful to elucidate the mechanism of antitumor as well.

In this paper, the experimental antitumor activity of Ce(IV) mitoxantrone complex is determined first, and

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Scheme 1. Structure of mitoxantrone.

then the different interaction model of mitoxantrone and Ce(IV)-mitoxantrone complex are studied by electrochemical, electrophoretic, and UV, circular dichroism (CD) spectrophotometric methods. The mechanism of action of the complex is analyzed.

Experiment

Reagents and Apparatus

Mitoxantrone was provided by the Zhenan Pharmaceutical Plant, China. $\text{Ce}(\text{SO}_4)_2 \cdot 4\text{H}_2\text{O}$ was purchased from Shanghai Reagents Supplying Station. Calf thymus DNA is the product of Sigma. ^3H -labeled thymidine was purchased from the Institute of Atomic Energy, China. The other chemical reagents are of analytical reagent (AR) grade. The concentrations of DNA and MX solution were determined spectrophotometrically at 260 nm ($\epsilon = 6600 \text{ cm}^{-1} \cdot \text{nucleotide}^{-1}$) and 682 nm ($\epsilon = 8360 \text{ cm}^{-1} \cdot \text{M}^{-1}$), respectively.

Absorption spectra were measured on a Shimadzu UV-365 spectrophotometer, the fluorescence spectra on a HITACHI RF-850 spectrofluorimeter. Circular dichroism spectra were recorded on a JASCO J-500C spectropolarimeter. The potentials were scanned on a JP-2 single-sweep oscillograph (Chengdu Instrument Factory, China) with a saturated calomel electrode (SCE) as reference electrode. Radioactivity was determined on PACKARD-Tri-Carb 2200CA liquid scintillation analyzer.

Procedure

Ehrlich ascites tumor (EAT) cells were diluted with RPMI 1640 medium to 1×10^6 cells/ml cell suspension. Comparing with 5×10^{-5} M MX, four different molar ratio of $[\text{Ce}(\text{IV})]/[\text{MX}]$: 0.5:1, 1:1, 1.5:1, and 2:1, were selected, respectively. 950 μl cell suspension and 50 μl drug were transferred in each cell of a 24-well plate. The plates were incubated at 37°C under CO_2 for 30 hr. The number of living and dead cells were determined with a counter counter. All survival studies were carried out in triplicate, and each experiment was repeated at least once. The death rate was calculated by the number of the death/the total number of cells. DNA synthesis was assessed by monitoring cellular incorporation of ^3H -labeled thymidine. Comparing with the same concentration of MX, when the molar ratio of $[\text{Ce}(\text{IV})]/[\text{MX}]$ is 0.5:1, 1:1, 1.5:1, or 2:1, three concentrations: 2×10^{-5} M, 2×10^{-6} M, and 2×10^{-7} M were selected, respec-

tively. 100 μl drug and 100 μl cell suspension were transferred in each well of a 96-well plate. The plates were incubated at 37°C under CO_2 for 1 h, and another 1 h after addition ^3H -thymidine ($0.6 \text{ u Ci well}^{-1}$). The cells were washed with salt water and then filtered through Millipore AP filters. The filters were dried under vacuum at 80°C , and associated radioactivity was determined after addition of a scintillation mixture. Every sample was triplicated in a parallely manner and average values were used. The effect on the incorporation of ^3H -thymidine were calculated by the value of counts per minute (CPM), showed by T/C% (CPM of drug/CPM of drug free).

Ce(IV) was added to the solution of DNA-MX, 50 mM tris-HCl, pH 7.0, as an electrolyte. When the ratio of concentration $[\text{Ce}(\text{IV})]/[\text{MX}]$ was 1:1 or 2:1, the potentials were scanned in a negative direction over the range of 0.0 to -1.0 V; absorption spectra and circular dichroism spectra were measured, respectively. Spectra of CD are expressed in terms of $\Delta\epsilon = \epsilon_L - \epsilon_R$ (molar CD coefficient).

For the gel electrophoresis, the standard reaction mixture contained 0.5 μg pBR322 DNA. Tris-HCl buffer, pH 7.5, was added to give a final volume of 20 μl . It was incubated at 37°C for 3 h in the presence or absence of MX and/or metal ions. The agarose gels were run on a horizontal slab gel electrophoresis apparatus. The gels were 0.8% agarose, and containing 0.5 u g ml^{-1} Ethidium Bromide (EB). The samples were electrophoresed on gels for 2–3 h at 150 V/40 mA. Pictures were taken with a Seagull Camera under UV light.

Results

1. Characteristics of Complexation

By adding Ce(IV) to a 1×10^{-4} M MX solution, absorption spectra and fluorescent spectra changed obviously, as shown in Figures 1 and 2. When the molar ratio of $[\text{Ce}(\text{IV})]/[\text{MX}]$ increased to 2:1, absorption spectra did not change obviously; the fluorescent peak decreased,

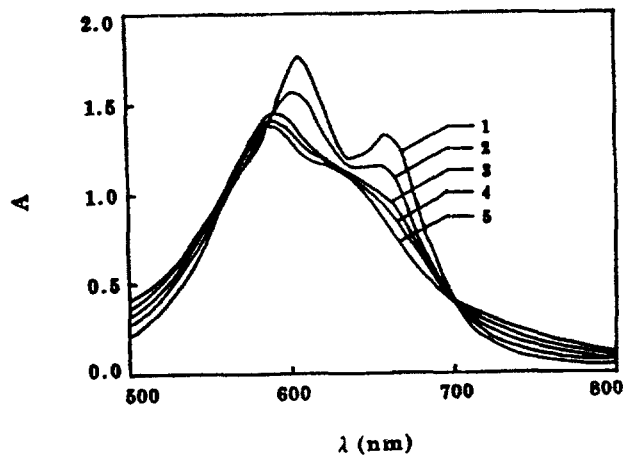


Figure 1. Absorption spectra for different molar ratios of Ce(IV)/MX, 1. 1.0×10^{-4} M MX, 2. 1:1, 3. 2:1, 4. 3:1, 5. 4:1. The samples were in deionized water, adjusted to pH 7.0.

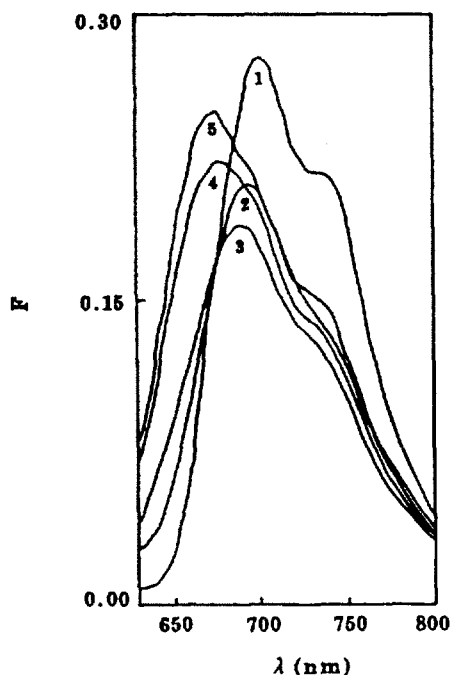


Figure 2. Fluorescence spectra for different molar ratios of Ce(IV)/MX, 1. 1.0×10^{-4} M MX, 2. 1:1, 3. 2:1, 4. 3:1, 5. 4:1. The samples were in deionized water, adjusted to pH 7.0.

blue shifted gradually and then increased obviously; an inflection point was found. This indicated that one molecule of drug can bind with two Ce(IV) ions. Adding Ce(IV) to 2×10^{-5} M MX solution, a reduction peak appeared at -0.80 V (vs SCE), which corresponds to the reduction potentials of the 9,10-quinone of MX [13]; a new peak appeared at -0.93 V (vs SCE), as shown in Figure 3. Because the $ip'_{0.80} + ip'_{0.93}$ is a constant and equal to the height of the reduction peak of MX, so the reduction peak at -0.93 V is corresponded to the reduc-

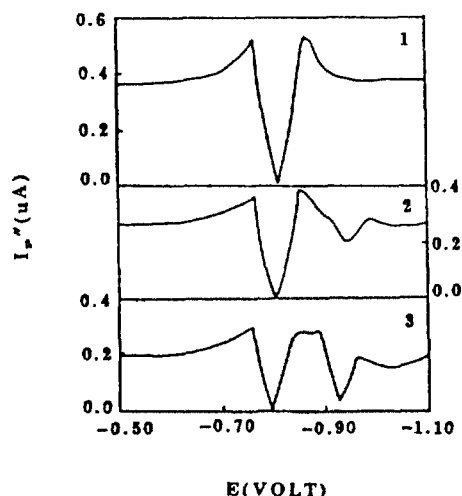


Figure 3. Linear sweep voltammogram of Ce(IV)-MX complex in 50 mM tris-HCl, pH 7.0. 1. 2.0×10^{-5} M MX, 2. 2.0×10^{-5} M MX + 2.0×10^{-5} M Ce(IV), 3. 2.0×10^{-5} M MX + 4.0×10^{-5} M Ce(IV).

Table 1. The Death Rate of EAT Cells Which Were Incubated with MX and Different Ratio of Ce(IV)/MX Complex.^a

MX	0.5:1	1:1	1.5:1	2:1
40.4%	45.7%	50%	40.2%	26.5%

^a The concentration of MX are the same, 5×10^{-5} M. The mean deviation is 1–3%.

tion potential of MX of the complex [14]. The reduction peak of the 9,10-quinone of MX was changed and split into two peaks; so Ce(IV) ions complex with oxygen atoms of the carbonyl function on C-9 and C-10. A stable chelate complex can be formed by chelating with oxygens of the hydroxyl groups at the 1,4 position and the carbonyl function on C-9 and C-10.

2. Antitumor Activity of the Ce(IV)-MX Complex

Survival Studies. The results of the cell culture are presented in Table 1. The complex exhibits a higher lethality than that of the free drug, especially the complex of 1:1-stoichiometry.

Thymidine Incorporation. ³H-labeled thymidine is the precursor of DNA synthesis. EAT cells were incubated with an incubation solution and by adding certain concentration of drugs, respectively; the effect of the drug on the DNA biosynthesis can be determined by the incorporation of thymidine into a cell [15]. The results of scintillation determination are tabulated in Table 2; it indicates that the incorporation of ³H-TdR is inhibited further by the complex than MX was, namely, the Ce(IV)-MX complex shows stronger inhibition ability on the DNA synthesis. Thus the Ce(IV)-MX complex may become a more potent antitumor drug than MX.

3. Study on the Interaction Model of the Ce(IV)-MX Complex with DNA

Absorption Spectra Study. Similar to other anthracycline-type drugs such as daunorubicin and adriamycin, MX possesses a planar geometry, the drug molecule intercalates between the base pairs of DNA, with the positively charged amine groups involved in an electrostatic interaction with neighboring phosphate groups [3].

Table 2. The Effect of MX and Ce(IV)-MX Complex on the Incorporation of ³H-TdR into an EAT Cell, Expressed at T/C%.^a

Compound	Concentration of MX / M		
	2.0×10^{-5} (%)	2.0×10^{-6} (%)	2.0×10^{-7} (%)
MX	13.5	43.0	75.3
Ce(IV)-MX(0.5:1)	9.4	40.0	88.1
Ce(IV)-MX(1:1)	9.6	39.3	62.1
Ce(IV)-MX(1.5:1)	7.5	33.3	71.3
Ce(IV)-MX(2:1)	8.3	34.5	58.5

^a The mean deviation is 1–5%.

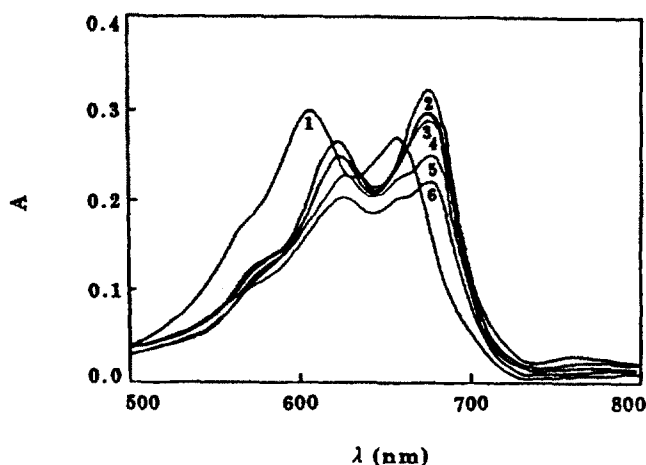


Figure 4. Absorption spectra for the system of MX-Ce(IV)-DNA in 50 mM tris-HCl, pH 7.0. 1. 2.0×10^{-5} M MX, 2. 2.0×10^{-5} M MX + 2.0×10^{-4} M DNA, 3. 2.0×10^{-5} M MX + 2.0×10^{-4} M DNA + 1.0×10^{-5} M Ce(IV), 4. 2.0×10^{-5} M MX + 2.0×10^{-4} M DNA + 2.0×10^{-5} M Ce(IV), 5. 2.0×10^{-5} M MX + 2.0×10^{-5} M Ce(IV) + 2.0×10^{-4} M DNA, 6. 2.0×10^{-5} M MX + 4.0×10^{-5} M Ce(IV) + 2.0×10^{-4} M DNA.

When MX binds to DNA by intercalation, the absorption peak of MX becomes red shifted, as shown in Figure 4. There is no absorption band of DNA in the visible region; the change of the absorption spectra in the visible region reflects the effect of binding environment on the drug molecule. By adding Ce(IV) to the solution of MX-DNA, the absorption spectra of this system practically did not change. However, if MX complexed with Ce(IV) first, then bound with DNA, the spectra should be obviously different from that of MX-DNA. We think that, when MX binds to DNA first, MX intercalated into the base pairs by the anthracenedione planar, oxygen atoms on the planar ring were packed by the base and bone of DNA phosphate; then adding Ce(IV), it can not complex with an oxygen atom; so, the absorption spectra of this system do not change. But if MX complexed with Ce(IV) first, this affected the chromophore on MX, leading to the change of the absorption peak, then they intercalate into DNA together leading to the red shift of the absorption peak of the Ce(IV)-MX complex. We can deduce that Ce(IV) ion complexes with oxygen atoms of ring planar by this as well.

Linear Sweep Voltammogram Study. Native DNA is not reducible at the mercury electrode because the stability of the intact double helix makes the reducible bases inaccessible to the electrode. In tris-HCl electrolyte, pH 7.0, only one reduction peak of MX appeared at -0.80 V (vs SCE) over the range of 0 to -1.0 V. When adding MX to the solution of DNA, the peak of the -0.80 V decreased to one very low in height, shown in Figure 5. We think that MX intercalates into the base pairs of DNA by the anthracenedione entity, and is not easily accessible to the electrode, thus causing the peak to decrease greatly. Adding Ce(IV) to 2×10^{-5} M MX

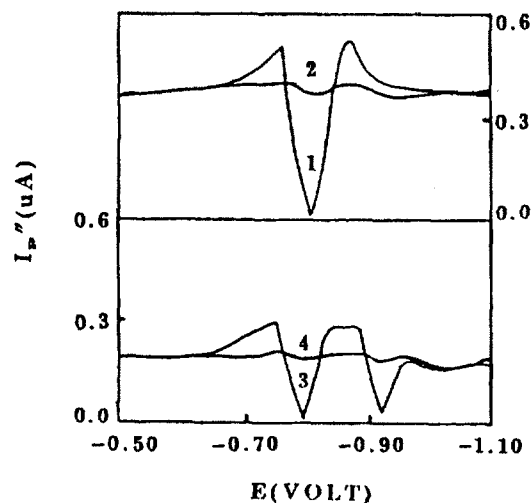


Figure 5. Linear sweep voltammogram of the system of MX-Ce(IV)-DNA in 50 mM tris-HCl, pH 7.0. 1. 2.0×10^{-5} M MX, 2. 2.0×10^{-5} M MX + 2.0×10^{-4} M DNA, 3. 2.0×10^{-5} M MX + 4.0×10^{-5} M Ce(IV), 4. 2.0×10^{-5} M MX + 4.0×10^{-5} M Ce(IV) + 2.0×10^{-4} M DNA.

solution, two reduction peak appeared at -0.80 V and -0.93 V (vs SCE), then mixed with solution of DNA, both the two reduction peaks of MX and the complex decreased gradually, even almost disappeared. This indicated that there is little free complex in the solution, both of them were intercalated into the bases of DNA by the planar aryl ring.

Circular Dichroism Study. Calf thymus DNA is a B-form helical conformation, while MX is not chiral; no characteristic of circular dichroism is displayed. The measurements of CD spectra are shown in Figure 6; the change

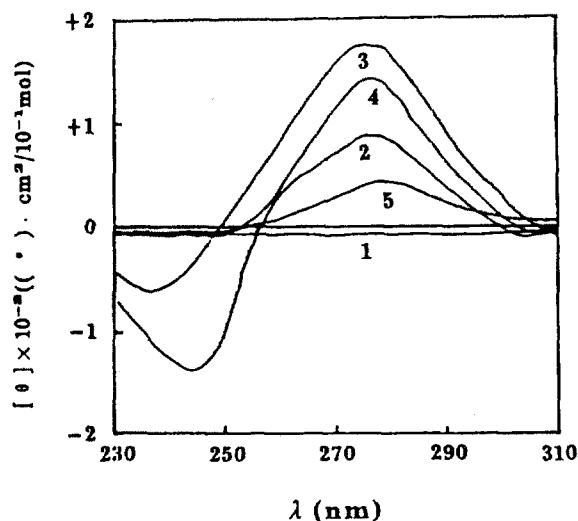


Figure 6. Circular dichroism spectra of the system of MX-Ce(IV)-DNA in 50 mM tris-HCl, pH 7.0. 1. 2.0×10^{-5} M MX, 2. 2.0×10^{-4} M DNA, 3. 2.0×10^{-5} M MX + 2.0×10^{-4} M DNA, 4. 2.0×10^{-5} M MX + 2.0×10^{-5} M Ce(IV) + 2.0×10^{-4} M DNA, 5. 2.0×10^{-5} M MX + 4.0×10^{-5} M Ce(IV) + 2.0×10^{-4} M DNA.

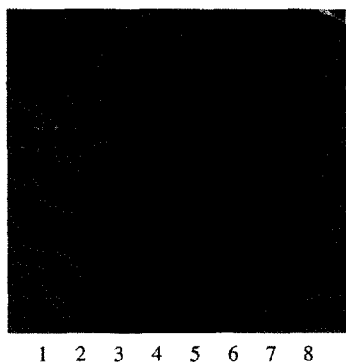


Figure 7. Agarose gel electrophoresis results of the system of MX-Ce(IV)-DNA in 50 mM tris-HCl, pH 7.0. 1. 0.5 μ g pBR322 DNA alone, 2. DNA + 1.0×10^{-5} M MX, 3. DNA + 1.0×10^{-5} M Ce(IV), 4. DNA + 2.0×10^{-5} M Ce(IV), 5. 1.0×10^{-5} M MX + DNA + 1.0×10^{-5} M Ce(IV), 6. 1.0×10^{-5} M MX + DNA + 2.0×10^{-5} M Ce(IV), 7. 1.0×10^{-5} M MX + 1.0×10^{-5} M Ce(IV) + DNA, 8. 1.0×10^{-5} M MX + 2.0×10^{-5} M Ce(IV) + DNA.

of CD spectra reflects the effect of drugs on the conformation of DNA. When MX binds with DNA, the positive band at 275 nm of DNA increases greatly, and the negative band at 245 nm decreases obviously; this indicates that binding with MX leads to the conformation transition of DNA from B to A [16]. When adding DNA to the solution of MX-Ce(IV), there is a red shift from 275 to 284 nm for a positive CD band and 237 to 245 nm for a negative CD band, both losing intensity seriously. The spectra of CD is very sensitive to the stack of bases, and the reduction of the pair number of bases will give rise to the highest positive peak decreasing gradually and red shift [17]. So, the change of CD spectra of the system suggests an additional change of the double-helical structure of DNA, and the pair of the bases may be broken.

Electrophoresis Study. As illustrated in lane 1 of Figure 7, pBR322 DNA shows two bands on agarose gel electrophoretograph, the faster moving band corresponds to the native form of covalently closed circular (CCC) DNA, the most slowly moving one to the open circular (OCC) form. Binding of MX with pBR322 by intercalating will give rise to unwinding of the super coiled conformation, reducing of the CCC band by single strand scission [18].

The presence of 2×10^{-5} M Ce(IV) alone (lanes 3,4), showed no effect on the conformation of DNA. When binding MX with pBR322, then Ce(IV) was added (lanes 5,6), the band of CCC reduced further. On this condition, Ce(IV) promoted the unwinding of CCC to OCC by MX. However, if binding MX with Ce(IV), then mixed with pBR322, the intensity of the CCC band was increased greatly, this indicates that the MX-Ce(IV) complex is favorable to preserve a more compact conformation of DNA.

Discussion

To sum up the preceding analysis results, we propose the following interaction model: MX binds with Ce(IV), then

intercalates into the base pairs of DNA together. Ce(IV) can form a weak interaction with oxygen of purine or pyrimidine, decreasing the negative charge from oxygen to hydrogen; or because of steric hindrance, the hydrogen bonds of base pairs are broken, and the number of pairs is reduced. In the meantime, owing to the high positive charge of a Ce(IV) ion, a compact stack is formed by the static electricity interaction of Ce(IV) with oxygen of bases and phosphates. Obviously, the complexation of Ce(IV) with MX gives rise to more changes on conformation and structure of DNA, changes the interaction model of MX with DNA; this is closely related to the change of its action on the survival of tumor cells.

To our surprise, both the complexes of Cu(II) and Ce(IV) show the strongest antitumor activity when the stoichiometry is 1:1; while increasing the coordination number of the ions, the activity of the complex decreases. Therefore, except for the change of the interaction model with cell targets, the complexation of the metal ions will also affect the metabolism processes of the drug, such as transferring in the body fluid and transporting through the membranes.

References

1. T. D. Shen Kenberg and D. D. Von Hoff, *Ann. Intern. Med.* **105**, 67 (1986).
2. M. A. Cornbleet, R. C. Stuart-Harris, and I. E. Smith, *Eur. J. Cancer Clin. Oncol.* **20**, 1141 (1984).
3. J. W. P. Lown, A. R. Morgan, S. F. Yen, Y. H. Wang, and W. D. Wilson, *Biochemistry* **24**, 4028 (1985).
4. J. Kapuscinski, Z. Daizynkiewicz, F. Traganos, and M. R. McLamed, *Biochem. Pharmacol.* **30**, 231 (1981).
5. J. Kapuscinski and Z. Daizynkiewicz, *Proc. Nat. Acad. Sci. U.S.A.* **83**, 6302 (1986).
6. A. L. Ellis, J. K. Randolph, B. R. Conway, and D. A. Gewirtz, *Biochem. Pharmacol.* **39**, 1549 (1990).
7. N. Chegini and A. R. Safa, *Cancer Lett.* **37**, 327 (1987).
8. C. Panousis and A. Garnier-Suilleot, *Nucleic Acids Res.* **8**, 1342 (1994).
9. P. Kolodziejczyk and A. Garnier-Suillerot, *Biochim. Biophys. Acta* **926**, 249 (1987).
10. P. Yang, H. F. Wang, F. Gao, and B. S. Yang, *J. Inorg. Biochem.* **62**, 137 (1996).
11. X. Y. Zhang, C. Q. Wang, J. T. Ma, and J. He, *Chem. Life* **13**, 29 (1993).
12. P. Yang, *Introduction of Bioinorganic Chemistry*, Xian Jiao Tong, University Press, Xian, People's Republic of China, 1989, p. 15.
13. B. Nguyen and P. L. Gutierrez, *Chem.-Biol. Interact.* **74**, 139 (1990).
14. X. X. Gao, *Polarographic Catalysis Wave*, Science Press, Beijing, People's Republic of China, 1991, pp. 142-144.
15. J. P. Zhang, *The Method and Technology of Cell Biology*, High Education Press, Beijing, People's Republic of China, 1990, pp. 155-159.
16. Z. X. Lu, *The Method of Circular Dichroism and Optical Rotatory Dispersion in Molecular Biology*, Science Press, Beijing, People's Republic of China, 1987, pp. 154-155.
17. W. B. Gratzer and E. G. Richards, *Biopolymers* **10**, 2607 (1971).
18. A. Someya and N. Tanaka, *J. Antibiot.* **32**, 839 (1979).