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Binding Affinity of Cu(II)–VP-16 (Etoposide) Complex and its Analogues to DNA and Hydroxyl Radical Generation During DNA Strand Breaks

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Abstract—Conformational effects and affinities of VP-16 (etoposide) and its derivatives to DNA in the presence of Cu(II) ion were examined by circular dichroic (CD) spectra. The Cu(II)/Cu(I) redox kinetics and the hydroxyl radical (·OH) generation from the Cu(II)-complexes were estimated by the stopped-flow kinetics. Based on the results, DNA-cleaving activity of Cu(II)-complexes of VP-16 has been shown to be related with binding affinity of the complex to DNA, Cu(II)/Cu(I) redox and ·OH generation, emphasising the mechanism of generated ·OH attack to DNA.

Introduction

Antitumor activity of VP-16 (etoposide) (Figure 1) has been suggested to be due to a covalent enzyme–DNA complex between the drug and topoisomerase II, which in turn produces double-strand DNA breaks in tumor cells.[1] Dimethoxyphenol ring of VP-16, on the other hand, has been implicated in its cytotoxicity by metabolic alterations to the dihydroxy and *o*-quinone derivatives catalyzed by either cytochrome P450, peroxidases and tyrosinase.[2] Copper ions may be particularly important in the biological capacity because they exist naturally in chromosomes.[3] Sinha et al. have shown that a metabolite dihydroxy-VP-16 promotes DNA damage in the presence of Cu(II) ion, indicating that hydroxyl radicals (·OH) are responsible for the DNA damage.[4] We have also found that ·OH can be generated during a redox-dependent complex formation between Cu(II) ion and VP-16 in the presence of molecular dioxygen (O_2), and suggested that the ·OH participates in the single- and double-strand breaks of DNA.[5,6] Cu(II) ion-induced DNA cleavage based on the results of metal complex formation and concomitant generation of ·OH which is thought to be formed by the redox systems of the copper centers, appears to be another possible and innovative mechanism for understanding the antitumor activity of VP-16. Therefore, copper redox cycle and ·OH generation mechanisms are two major determinants involved in the observed Cu(II)–VP-16-induced DNA damage.

In this study we examine the circular dichroic (CD) spectra to analyze the interactions of VP-16 and its derivatives (Figure 1) with natural DNA and the kinetics of Cu(II)-induced ·OH generation from VP-16 related compounds in the presence of DNA to understand in more detail the DNA damage mediated by Cu(II)-induced activation of VP-16.

Results and Discussion

We have reported previously that VP-16 and its derivatives induce single- and double-strand breaks in the

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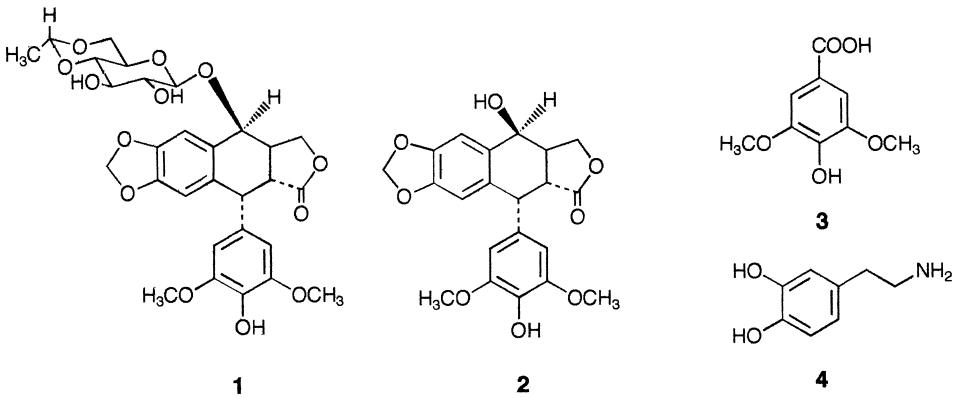

Figure 1. The structures of **1**, **2**, **3** and **4**.

pBR322-*Bgl*I fragments[6] in the presence of Cu(II) ions, and that Cu(II) ion strongly mediates the oxidation of VP-16 to the benzoquinone type compounds through a copper redox mechanism.[5] As shown in Table 1, the interactions of VP-16, DEPD and SA with Cu(II) ion induced the formation of both open circular and linear forms in the supercoiled pBR322 DNA. The system of 500 μM Cu(II) ion and 500 μM SA cleaved DNA strand more extensively than the system of VP-16 or DEPD plus 500 μM Cu(II) ion did. Neither VP-16, DEPD and SA at the indicated concentrations nor Cu(II) (500 μM) alone induced DNA strand breaks. It has been reported that L-DOPA in presence of Cu(II) ion is capable of causing DNA strand breakage in vitro and in which the generation of ·OH radicals is observed.[9] Recently dopamine (DOPM), one of the catecholamine formed by the decarboxylation of L-DOPA, has been reported to cause extensive base modification in DNA in the presence of H_2O_2 and traces of Cu(II) ion.[10] In support of the results, we also observed ·OH generation from 2 mM DOPM in the presence of 500 μM Cu(II) ion at pH 7.8 by the salicylate hydroxylation assay (data not shown). Thus, we compared the DNA strand breaks induced by SA–Cu(II) to that of a DOPM–Cu(II) system. After 10 min of incubation at 25 °C, 69% of the supercoiled DNA was converted to open circular and linear forms, demonstrating that the Cu(II)–DOPM-induced DNA strand breaks occur very quickly, when compared that of Cu(II)–SA system (Figure 2 and Table 1). With a longer time of incubation for 30 min, DNA completely disappeared resulting in a smear, suggesting that 12.5 μM DOPM in the presence of 0.36 μM Cu(II) causes extensive DNA double strand breaks, resulting in DNA degradation. These results clearly show an occurrence of structure–activity relationship between SA and DOPM. CD measurements on the binding of the Cu(II)-complexes to the double-helical structure of DNA were carried out for understanding in more detail the conformational features involved in the DNA strand breaks under the conditions used. The spectroscopic titration of the Cu(II)-complexes with calf thymus DNA was followed in the ultraviolet region where DNA has B-form conformation as evidenced by a pair of conservative bands.[11] The effect of 0.17 mM Cu(II)-complexes on the CD spectrum between 230 and 300 nm of 0.17 mM calf thymus DNA is shown in Figure 3A. The band at 275 nm in the CD spectrum was perturbed progressively on increasing the concentration of Cu(II)-

Table 1. Estimation of the cleavages in pBR322 produced by VP-16, DEPD, SA and DOPM in the presence of Cu(II) ion and O_2

	Cleaving activity (%)[a]	Conditions[b]
VP-16 10 mM + Cu(II) 0.5 mM	38.4 ± 9.5	37 °C, 3 h
VP-16 0.5 mM + Cu(II) 0.5 mM	11.8 ± 2.6*	37 °C, 3 h
DEPD 4.5 mM + Cu(II) 0.5 mM	70.8 ± 5.6*	37 °C, 3 h
DEPD 0.5 mM + Ca(II) 0.5 mM	19.8 ± 1.0*	37 °C, 3 h
SA 0.5 mM + Cu(II) 0.5 mM	76.0 ± 2.2*	37 °C, 1 h
SA 12.5 μM + Cu(II) 0.36 μM	0.6 ± 1.1*	25 °C, 10 min
DOPM 12.5 μM + Cu(II) 0.36 μM	68.6 ± 2.1*	25 °C, 10 min
	100 ± 0	25 °C, 30 min
Cu(II) 0.5 mM	1.9 ± 0.2	37 °C, 3 h

[a]Each value is the mean ± SD for five independent experiments, as described previously.[7]
[b]Reactions were carried out in 20 mM TA buffer (pH 7.8).
*Significant difference from the corresponding value; $p < 0.001$.

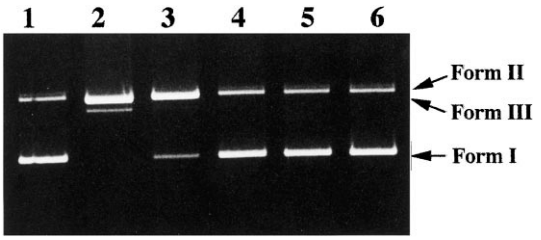

Figure 2. pBR322 DNA cleavages by **3** and **4** in the presence of Cu(II) ion. pBR322 DNA (0.25 μg) was incubated at 25 °C in 20 mM TA buffer (pH 7.8). Lane 1, pBR322 DNA 0.25 μg; Lanes 2 and 3, pBR322 DNA reacted with 12.5 μM **4** and 360 μM Cu(II) ion for 30 and 10 min, respectively; Lanes 4 and 5, pBR322 DNA reacted with 12.5 μM **3** and 360 μM Cu(II) ion for 30 and 10 min, respectively; Lane 6, pBR322 DNA reacted with 360 μM Cu(II) ion for 30 min.

complexes, as demonstrated by a Lineweaver–Burk plot of δ[θ], the mean residue ellipticity at the maximum (275 nm), as a function of the concentration of added complexes (Figure 3B). The observed limiting ellipticity value, $\delta[\theta]_{max}$, and the DNA affinity constant value, *Km*, for each Cu(II)-complex are summarized in Table 2. The association of Cu(II)–DOPM complex to DNA induced much greater optical activity than those of other Cu(II)-complexes at equivalent concentration. On the other hand, Cu(II)–SA complex gave higher affinity for calf thymus DNA than other Cu(II)-complex systems did. In contrast to these drastic changes in the positive CD band, slight changes were observed in the negative band around 245 nm for the Cu(II)-complexes examined. Because of the chelate formation with guanine of G–C pairs, the hydrogen bonds of the double helical DNA may be weakened and thus enhance the fluctuational opening of the DNA double-helical structure.

The ·OH formation has already been observed in the dihydroxy VP-16-Fe(III) system in the presence of hydrogen peroxide.[12] In the previous paper, we reported that the Cu(II)–SA system displayed complicated electron spin resonance (ESR) signals due to the 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO)-·OH adduct (DMPO/·OH), together with unidentified small signals, although SA or Cu(II) ion alone gave no signals.[5] Even if such DNA-associated ·OH is generated, however, ·OH is very short-lived and tends to react at near diffusion-controlled rates with DNA. Therefore, it is hard to estimate the level of ·OH in the system by ESR data. To determine ·OH level, a bleaching of pNDA has been used as an indicator of ·OH.[13] Owing to the large extinction coefficient of pNDA ($34{,}800\ M^{-1}\ cm^{-1}$ at 445 nm in 20 mM TA buffer solution, pH 7.8; present study), it is possible to measure accurately very small changes of ·OH concentration. Radiolytic and photolytic studies revealed that pNDA is not oxidized by superoxide anions (O_2^-) or singlet oxygens ($^1O_2$), whereas the reaction with ·OH is extremely rapid ($1.25\times10^{10}\ M^{-1}\ s^{-1}$).[14] Since it is known that the intermediates formed during the interaction of ·OH and pNDA can undergo further reaction resulting in the recovery of pNDA but this recovery is slow in aqueous solution,[15] our proposed method allows the quantification for the generation of ·OH by assuming a 1:1 stoichiometry for the radical bleaching.[13] In the present investigation we measured the bleaching rates of pNDA

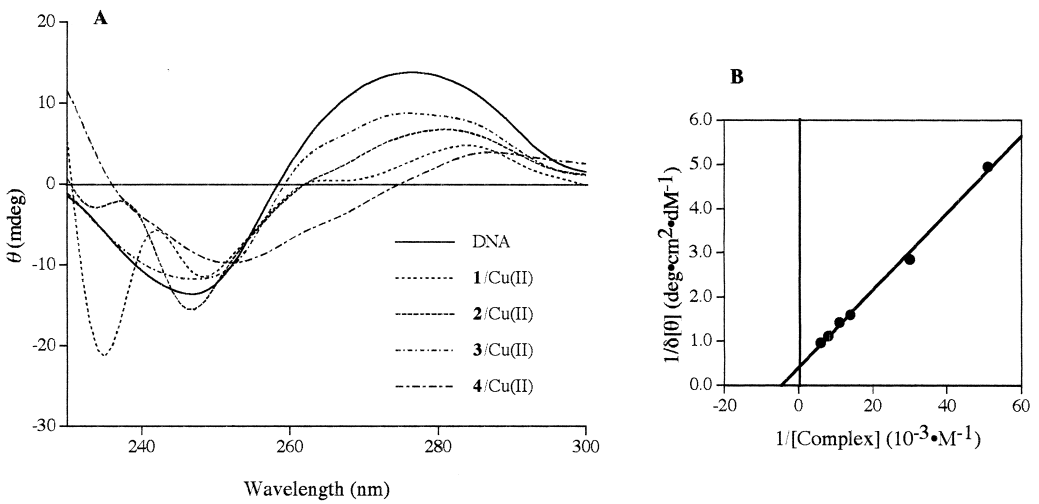

Figure 3 (**A**). CD spectra of calf thymus DNA in solution containing the Cu(II)-complex. The solid line represents the B-type DNA (0.17 mM) in 20 mM TA buffer. The other solutions contained calf thymus DNA (0.17 mM) plus each Cu(II)-complex (0.17 mM) in the same buffer solutions. (**B**) Lineweaver–Burk plot of CD spectral changes of calf thymus (0.17 mM) upon titration of Cu(II)–**3** complex (0.17 mM).

Table 2. Estimation of the apparent limiting ellipticity value, $\delta[\theta]_{max}$, and the apparent binding affinity constant, K m, of Cu(II)-complexes to calf thymus DNA[a]

	$\delta[\theta]$max(deg/cm^2 dmol^{-1})	Km ($\times 10^{-5}$ M^{-1})
Cu(II)–VP-16	3.25 ± 0.91	19.5 ± 4.6
Cu(II)–DEPD	3.07 ± 1.00	12.8 ± 0.4
Cu(II)–SA	1.66 ± 0.11	5.5 ± 0.7
Cu(II)–DOPM	25.8 ± 4.16	78.6 ± 13.4

[a]Each value is the mean ± SD for three independent experiments, as described previously.[7] CD measurements were carried out in 20 mM TA buffer (pH 7.8).

by the stopped-flow spectroscopy for evaluating ·OH generation from Cu(II)-complexes in the presence of DNA. The pNDA bleaching reaction, of which a typical measurement is represented in Figure 4, proceeds in two steps as follows: first step, ·OH radicals react with pNDA and consumed, and second step, the formed active species disappear.[16] Therefore, the bleaching monitored at 445 nm could be thought due to attack by ·OH radicals were generated in the reaction of Cu(II)-complex with O_2. Since the maximum change in the concentration of pNDA was less than 5% of its initial concentration, the consumption of ·OH radicals, i.e. ·OH generation-rate was followed by first-order kinetics under the conditions used. The remarkable ·OH generations were observed from the rates of Cu(II)–SA and –DOPM systems, as shown in Table 3.

As suggested in the previous paper, Cu(II) ion bound with VP-16, DEPD or SA is reduced and the resulting Cu(I) ion or its complexes would then react with O_2 during ·OH generation process.[5,17] In order to clarify

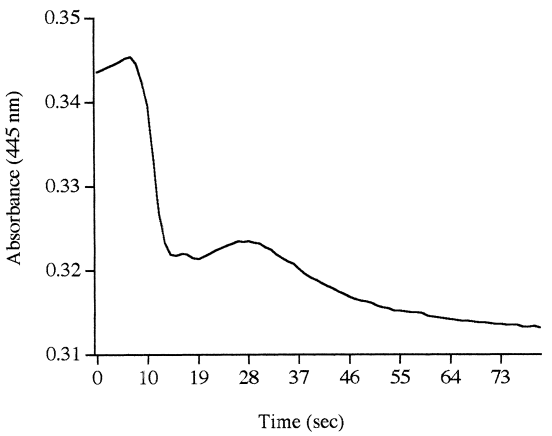

Figure 4. Changes in absorption of 445 nm in 4×10^{-5} M pNDA solution reacted with 2 mM **4** plus 0.5 mM Cu(II) ion in 20 mM TA buffer (pH 7.8) at 37 °C in the presence of 25 μg mL^{-1} calf thymus DNA.

Table 3. First-order rate constants for ·OH generation from Cu(II)-complex in the presence of calf thymus DNA[a]

Complex	Rate constants k_1 (sec^{-1})(n)[b]
SA 2 mM + Cu(II) 0.5 mM	$1.24 \pm 0.34\times10^{-2}$(8)
DOPM 2 mM + Cu(II) 0.5 nM	$1.12 \pm 0.07\times10^{-2}$(3)

[a]The stopped-flow measurements were carried out at 37 °C in 20 mM TA buffer (pH 7.8) in the presence of pNDA (4×10^{-5} M) and calf thymus DNA (25 g mL^{-1}).
[b]Each value is the mean ± SD for n independent experiments.

Table 4. First-order rate constants of Cu(I)–DNA formation from Cu(II)–SA or –DOPM complex by the reaction with DNA[a]

Complex	First-order rate constants k_1 (sec^{-1})(n)
SA 500 μM + Cu(II) 500 μM	$5.66 \pm 0.60\times10^{-3}$(4)
SA 500 μM + Cu(II) 75 μM	$1.64 \pm 0.25\times10^{-3}$(4)
DOPM 500 μM + Cu(II) 500 μM	$1.39 \pm 0.08\times10^{-1}$(5)
DOPM 500 μM + Cu(II) 75 μM	$6.65 \pm 0.03\times10^{-2}$(5)

[a]The stopped-flow measurements were carried out at 37 °C in 20 mM TA buffer (pH 7.8) in the presence of 250 μg mL^{-1} calf thymus DNA. Each value is the mean ± SD for n independent experiments.

the importance of a Cu(II)/Cu(I) redox mechanism as the initial reaction in the ·OH generation from these Cu(II)-complexes, we compared the time courses for the reduction of Cu(II) to Cu(I) in the presence of DNA using SA and DOPM. It has been known that Cu(II)-induced oxidation of certain compounds such as catechols was enhanced by orders of magnitude in the presence of DNA.[18] Furthermore, DNA-bound Cu(II) ion can still undergo cyclic redox reaction,[19] suggesting that both SA and DOPM are oxidized by a Cu(II) redox mechanism. Cu(I) ion has been known to form a very strong complex with DNA.[20] Thus obtained first-order rate constants of DNA-Cu(I) formation by the oxidation of SA and DOPM are summarized in Table 4. The strong absorbance change at 300 nm, which is due to the formation of DNA-Cu(I), is explained by the high affinity of DNA with Cu(I) (stability constant $K = 2\times10^9$ M^{-1}),[21] which enables the electron-transfer equilibria between Cu(II) and SA or DOPM as electron donors, even if thermodynamically unfeasible.

Conclusion

DNA breakage activity of Cu(II)-complexes of VP-16 and its derivatives has been revealed to correlate with ·OH generation, the binding affinity of the complex to DNA, and Cu(II)/Cu(I) redox rates. These results could

add a further support to the hypothesis that ·OH radical attach to DNA is one of the major factors contributing to the cytotoxicity by VP-16 in vivo.

Experimental

Materials

pBR322 DNA (0.51 mg/mL) and calf thymus DNA were purchased from Takara and Sigma, respectively. A stock solution of calf thymus DNA (1 mg/mL) was prepared by slow stirring at 25 °C for 2 days. VP-16, syringic acid (SA) and dopamine (DOPM) were obtained as commercial products Sigma, Tokyo Kasei, Nakalai Tesque, respectively. 4′-Demethylepipodophyllotoxin (DEPD) was prepared as described previously.[7] *N*,*N*-Dimethyl-*p*-nitrosoaniline (pNDA) from Nakalai Tesque was dissolved in the purified water as described below. All other reagents were of analytical-reagent grade and were used without further purification. Distilled water used in all solutions was treated with a MilliQ-II water purification system (Millipore).

DNA cleavage reactions. pBR322 DNA (0.25 μg) were incubated in a reaction mixture containing Cu(II) ion (0.5 mM $CuCl_2$) plus VP-16, DEPD, SA or DOPM in 20 mM Tris–acetate (TA) buffer (pH 7.8) at 25 or 37 °C in water-bath. Ligand concentrations used are given in Table 1 and Figure 2. Reactions were started by addition of the ligand and stopped by addition of 2 μL of a terminating agent containing 40% sucrose and 0.25% bromophenol blue. pBR322 DNA reacted with Cu(II)-complexes were electrophoresed on 0.8% agarose-gel and the induced DNA breaks were evaluated by analyzing the gels using GDS7500 gel documentation system (UVP) after staining with ethidium bromide (0.5 μg/mL).

CD measurements. CD spectral measurements were made at 25 °C in 1 cm cells with a Jasco J-720WI spectropolarimeter (Japan Spectroscopic Co.). Each Cu(II)-complex solution (1 mM) for 1:1 molar ratio of ligand and Cu(II) ion was prepared and the appropriate volumes of the complex solution were added in 2 mL of 20 mM TA buffer (pH 7.8) and 10 mM NaCl containing 0.2 mM calf thymus DNA. The calibration of the CD unit was routinely checked with 0.06% (w/v) aqueous solutions of ammonium *d*-10-camphorsulphonate (λmax: 290.5 nm) (Wako Pure Chemical Ind.) in a quartz cell of path length 1 cm. The molar ellipticity, [θ], was calculated from the observed ellipticity in degrees by the equation, $[\theta] = \theta/10cl$, where θ is the observed ellipticity in mdeg, c is the concentration expressed in mol/L and l is the path length in cm; the unit of [θ] is degree/cm^2/dmol. All reported spectra are expressed as the average of three repetitive scans.

Reaction rate methods. Kinetic experiments were performed under pseudo-first-order conditions on a RA-401 stopped-flow apparatus (Otsuka Electronics). The sample handling unit was fitted with two drive syringes that are mounted inside a thermostated water-bath compartment. Calf thymus DNA solution (0.5 mg/mL) containing the ligand (SA and DOPM) solution (1 mM) was prepared as a solution in 40 mM TA buffer (pH 7.8). Cu(II) ion was prepared as solutions at 75 and 500 μM in the purified distilled water. Both solutions were injected simultaneously at 37 °C, and the formation of the Cu(I)–DNA complex was monitored by the change in UV absorption at 300 nm. For ·OH generation kinetics, calf thymus DNA solution (0.5 mg/mL) containing the ligand solution (4 mM) was prepared as a solution in 40 mM TA buffer (pH 7.8). A solution containing Cu(II) ion (500 μM) and pNDA (8×10^{-5} M) was prepared. Both solutions were mixed at 37 °C, and the absorption change was monitored at 445 nm which is a maximum absorption wavelength of pNDA as acceptor of ·OH under the conditions used. The flow-through mixing cell was of 1 cm optical path length. First-order curve fitting and rate constants based on the Gauss–Newton method were calculated through software provided by Otsuka Electronics.[8] Statistical analysis was performed with Student's *t*-test for comparison between the DNA cleaving activities, and probability of 0.05 or less was considered to be significant.

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