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Site-specific DNA cleavage by Cu(II) complexes of podophyllotoxin derivatives

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Abstract—Site-specific DNA cleavage in the presence of Cu(II) complexes of podophyllotoxin derivatives was investigated with a modified Sanger sequencing method. Cu(II) complexes of 4′-demethylepipodophyllotoxin (DEPD) and syringic acid (SA) cleaved M13mp18 single-strand DNA site-specifically at both cytosine (C) and guanine (G) positions in the GC rich regions and C position, respectively, at pH 7.8. The apparent binding constants of calf thymus DNA–Cu(II) complexes estimated by the differential UV-absorption spectra revealed that both Cu(II)–VP-16 and –DEPD complexes bind to DNA more strongly than does the Cu(II)–SA complex.

Key words: podophyllotoxin derivatives; metal complexes; DNA cleavage; DNA binding; sequencing; hydroxyl radical

It is known that a currently useful antitumor drug, VP-16 (etoposide*), an analog of podophyllotoxin (PD), induces both single- (ss) and double-strand (ds) DNA breaks in tumor cells (HeLa and L1210) [1, 2]. Several mechanisms of DNA cleavage by VP-16, such as poisoning type II topoisomerase [3], O-demethylation [4, 5] and formation of semiquinone free radicals, have been proposed [6, 7]. Sinha *et al.* [8, 9] have shown that VP-16 and its metabolites catalyze the generation of hydroxyl radicals in the presence of Fe(III) ion and induce DNA damage. Previously, we also reported that PD and its related compounds, VP-16, 4′-demethylepi-PD (DEPD) and syringic acid (SA), cleave plasmid DNA (ColE1) in the presence of metal ions (Fig. 1) [10]. The 1:1 complexes of Cu(II): or Fe(II):ligand converted the supercoiled DNA into the open circular and/or the linear forms. In addition, based on the results of metal complex formation and concomitant generation of hydroxyl radicals detected by the EPR-spin-trapping method, we proposed a new mechanism of the hydroxyl radical dependent-DNA cleavage induced by the metal complex formation. In the present communication, we report base specificity in the site-specific DNA cleavage by the Cu(II) complexes, together with evidence of DNA binding of the complexes.

Materials and Methods

Materials. The M13mp18 ss- and dsDNA were purchased from the Takara Shuzo Co. Calf thymus DNA was from the Sigma Chemical Co. All chemical compounds used were obtained as described in the previous report [10]. Electrophoresis-grade agarose was used (TAKARA L03). Distilled water employed in all the solutions was deionized and filtered (Millipore).

DNA cleavage. The M13mp18 ss- or dsDNA was incubated in 30 μL of a reaction mixture containing various concentrations of metal ions plus SA, DEPD, PD or VP-16 in 20 mM Tris-acetate buffer, pH 7.8, at 37°. Ligands and metal ion concentrations are given in the legends to the figures. Reactions were started by the addition of the ligand and stopped after 60 min by the addition of 2.5 μL of a terminating agent containing 20 mM Tris-acetate buffer, 50% glycerol, 3 mM EDTA and 0.1% bromophenol blue. Aliquots of the samples were loaded on a 1% agarose gel, and the remaining samples were used for the sequence determinations.

Agarose gel electrophoresis. The induced breaks for M13mp18 ssDNA were analyzed on 1.0% agarose-gel electrophoresis with 40 mM Tris-acetate/1 mM EDTA as a running buffer. The gels were stained with ethidium bromide and photographed with short wavelength UV light (303 nm).

Sequence determination. The sequence reactions, used as markers, were performed with a Deaza Sequenase® (Version 2.0) DNA sequencing kit (United States Biochemical) and [α-^{35}S]dATP (18.5 TBq/mmol) (NEN Research Products) according to standard method [11]. The radioactive bands were detected by exposure of the dried gels to New RX X-ray film (Fuji Film Co.) at room temperature overnight. Synthesis of strands complementary to the modified M13mp18 ssDNA after reaction under the various conditions was carried out using T7DNA polymerase, an M13 primer (40-mer), and four deoxynucleotides (dATP, dCTP, dGTP and dTTP) [12].

Densitometry. For analysis of sequencing patterns, autoradiographs were scanned on a Shimadzu CS-9000 dual-wavelength flying-spot scanner.

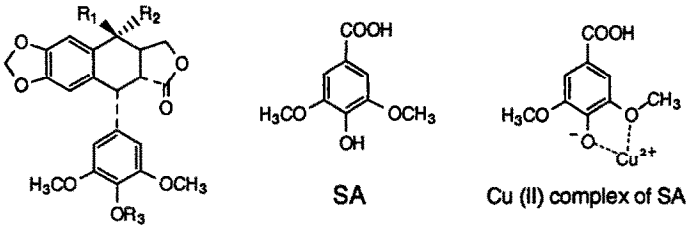
Differential spectroscopy. UV-absorption spectra were recorded on a JASCO U-best 50 UV/visible spectrometer at 37°.

Results and Discussion

Neither 1.67 mM SA nor 0.67 mM Cu(II) ion alone induced any strand breakage in M13mp18 ssDNA under the conditions used (Fig. 2, lanes 2 and 3). However, when 1.67 mM SA in the presence of 0.33, 0.67 or 1.34 mM Cu(II) was reacted with DNA for 60 min at 37°, DNA was degraded significantly (Fig. 2, lanes 4–6). The amounts of DNA fragments produced in the reactions were proportional to the increased ratios of Cu(II) to a fixed concentration of SA (1.67 mM). The same results were also obtained for the Cu(II) complexes of DEPD or VP-16 (data not shown). The order of cleavage rates was as follows: DEPD > SA > VP-16.

To find base-specific inhibitions for DNA polymerase action, M13mp18 ssDNA was treated with a mixture of 0.67 mM Cu(II) and 1.67 mM SA. The resulting fragments were used as templates for an *in vitro* DNA synthesizing system [12]. This assay was based on the premise that

* Abbreviations: etoposide, 4′-demethylepipodophyllotoxin-9-(4,6-*O*-ethylidene-β-D-glucopyranoside); DEPD, 4′-demethylepipodophyllotoxin; SA, syringic acid; PD, podophyllotoxin; C, cytosine; G, guanine; ss, single-strand; and ds, double-strand.



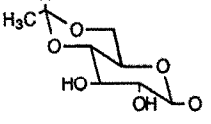
Compound	R_1	R_2	R_3
PD	H	OH	CH_3
DEPD	OH	H	H
VP-16		H	H

Fig. 1. Structures of PD, DEPD, VP-16 and SA and the putative Cu(II) complex of SA.

either binding or interaction of a metal complex with DNA inhibits DNA synthesis by polymerization with the M13 primer DNA. The autoradiogram obtained and a densitometer scan of it are shown in Figs. 3 and 4. Short fragments with non-base-specific breakages in M13mp18 ssDNA are shown (Fig. 3, lane 1; see also Fig. 6A). On the other hand, for the longer DNA fragments, site-specific cleavages were found, especially on most G positions in M13mp18 ssDNA, i.e. most C positions on the complementary DNA strand are responsible for DNA strand scission by SA–Cu(II) complex (Fig. 3, lane 1; see also Figs. 4 and 6A). In addition, the base specificity of strand breaks in M13mp18 ssDNA treated with DEPD-Cu(II) complex was investigated (Figs. 5 and 6). As shown in Fig. 6, the positions inhibiting DNA polymerase action along the DNA template were found at G and C in the GC-rich regions that correspond to the region of bases 218–310 of M13mp18 DNA. These results suggest that the primary sequence of M13mp18 DNA may affect the binding and interaction of Cu(II) complexes with SA or DEPD.

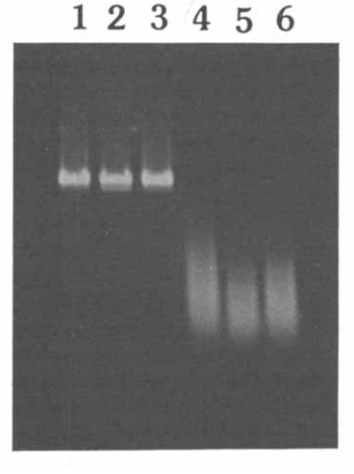


Fig. 2. Agarose gel electrophoretic pattern of DNA degradation by SA in the presence of Cu(II) ion. M13mp18 ssDNA (3 μg) was incubated for 1 hr at 37° as follows: (a) DNA control (lane 1); (b) SA (1.67 mM) alone (lane 2); (c) Cu(II) ion (0.67 mM) alone (lane 3); (d) SA (1.67 mM) plus Cu(II) ion at 0.33 mM (lane 4), 0.67 mM (lane 5) or 1.34 mM (lane 6).

Binding of SA, DEPD or VP-16 with calf thymus DNA in the presence of Cu(II) ion was studied by differential UV-absorption spectroscopy at 225–320 nm at 37°. During titration of 15 μM SA, DEPD or VP-16 alone or their complexes at a concentration of 1.5, 7.5 or 15 μM Cu(II) with 2.75 mM calf thymus DNA, the absorption changes at 260 nm were monitored. When Cu(II)/SA or Cu(II)/DEPD were 0.5 or 1.0, the absorptions at around 260 nm were decreased most extensively upon addition of DNA, as shown in Fig. 7. Such Cu(II) complex-induced reductions of DNA absorbance are due to the interactions of DNA with the complex. The absence of isosbestic points suggests that the interactions are geometrically heterogeneous. A Lineweaver–Burk plot describing the binding affinities of Cu(II) complexes of SA, DEPD or VP-16 to DNA, with the absorbance at 260 nm versus DNA concentrations, is shown in the inset of Fig. 7. Apparent binding constants (K_m) were derived from the best fit to data points of each plot. Both Cu(II)–VP-16 and -DEPD complexes gave higher affinity for calf thymus DNA [K_m: $2.59 \times 10^{-4}\,M^{-1}$ for Cu(II)–VP-16, $3.92 \times 10^{-4}\,M^{-1}$ for Cu(II)–DEPD] than did the Cu(II)–SA complex system (K_m: $2.05 \times 10^{-3}\,M^{-1}$). These data indicate that the binding affinity of Cu(II) complexes to DNA may affect the cleavage positions on DNA strands. The affinity characteristic of Cu(II)–VP-16 complex to DNA may relate to the stability of a ternary complex among DNA, Cu(II) and VP-16, as observed by the relatively low DNA cleavage [10]. Further, the contribution of the sugar moiety of VP-16 [1] should be considered in the binding of DNA with the complex. Although the exact mechanism for the cytotoxic properties of PD and its derivatives remains unclear, it often has been proposed that these molecules stimulate DNA breakage by interacting with topoisomerase II and DNA to form cleavable complexes; this event is seen as a primary step leading to cell death [13]. In a previous study we demonstrated that these compounds generate active oxygen species such as hydroxy radicals ·OH, which in turn induce the nicking of plasmid DNA [10], when they bind to biometal ions in the presence of molecular oxygen. The

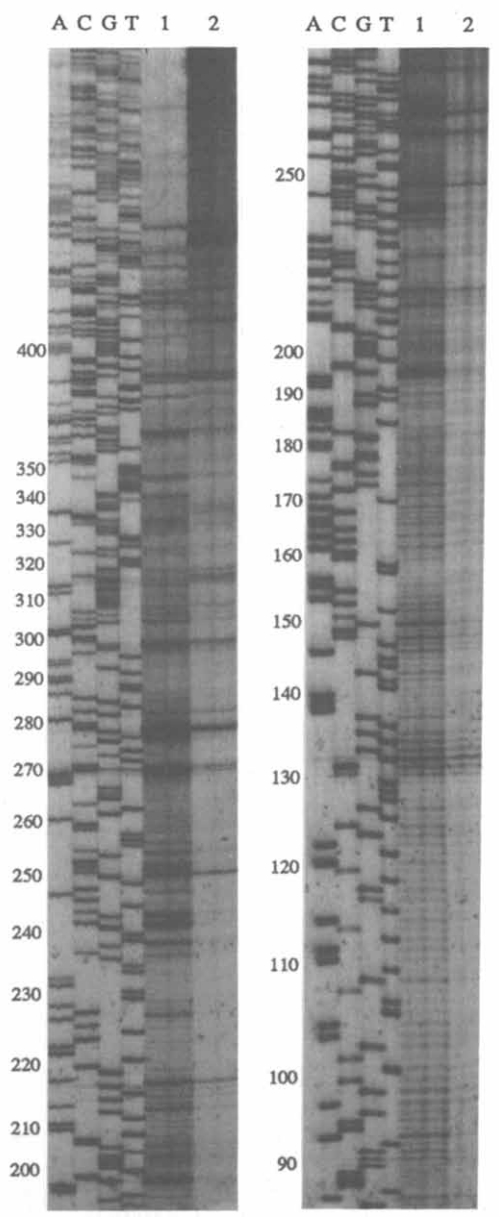


Fig. 3. Autoradiogram of the sequencing gel showing the positions of the induced stops with (lane 1) or without (lane 2) Cu(II) (0.67 mM)–SA (1.67 mM) complex in the template M13mp18 ssDNA. Lanes A, C, G and T refer to M13mp18 DNA dideoxy sequencing reaction.

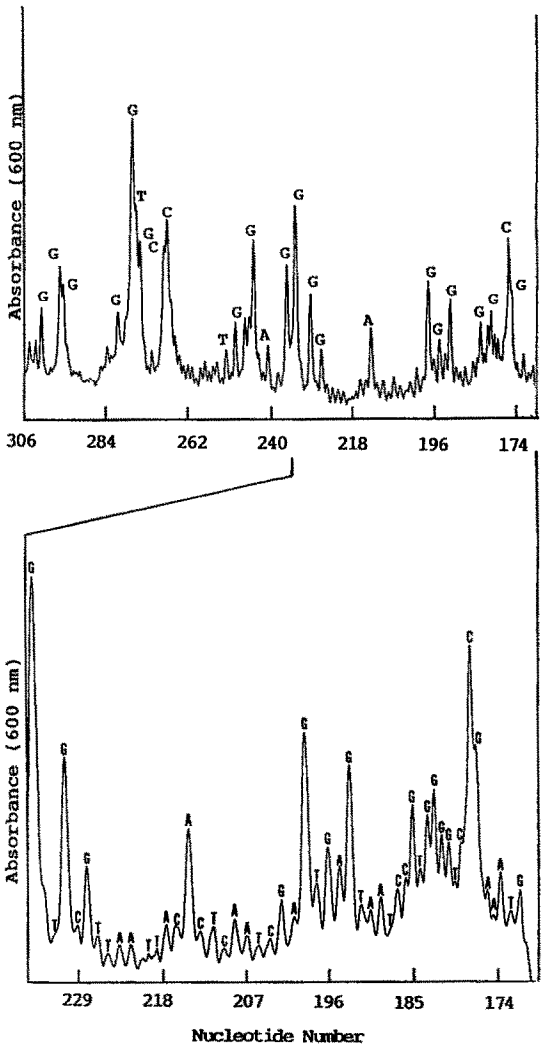


Fig. 4. Densitometer scan of the autoradiogram in Fig. 3.

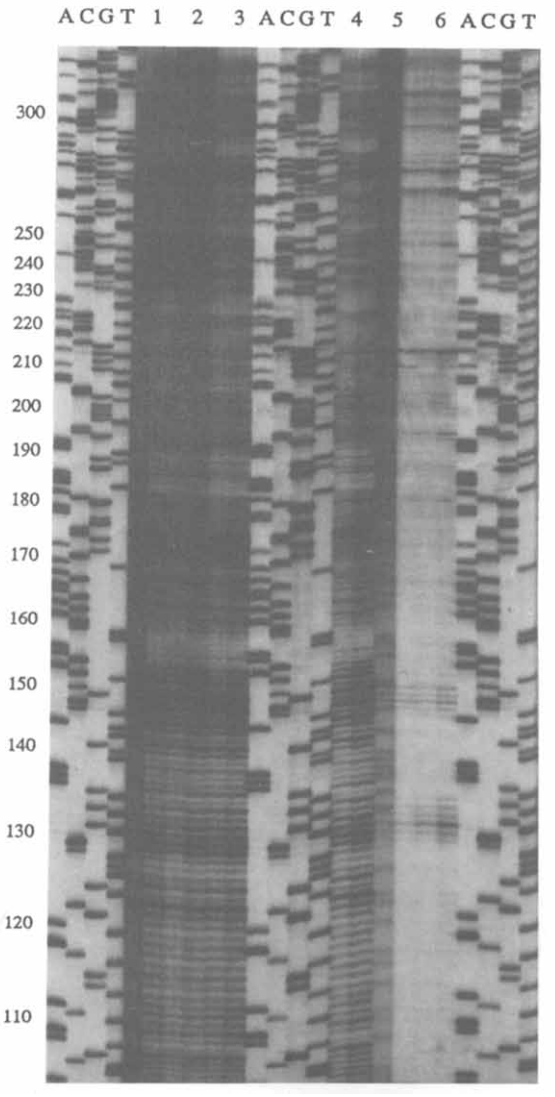


Fig. 5. Autoradiogram of a modified dideoxy sequencing gel showing the positions of the induced stops with DEPD (1.67 mM) plus Cu(II) ion at 0.6 mM (lane 1), 0.5 mM (lane 2), 0.47 mM (lane 3), 0.43 mM (lane 4) or 0 mM (lane 5), or with Cu(II) ion (0.5 mM) alone (lane 6) in the template M13mp18 DNA. Lanes A, C, G and T refer to M13mp18 DNA dideoxy sequencing reaction.

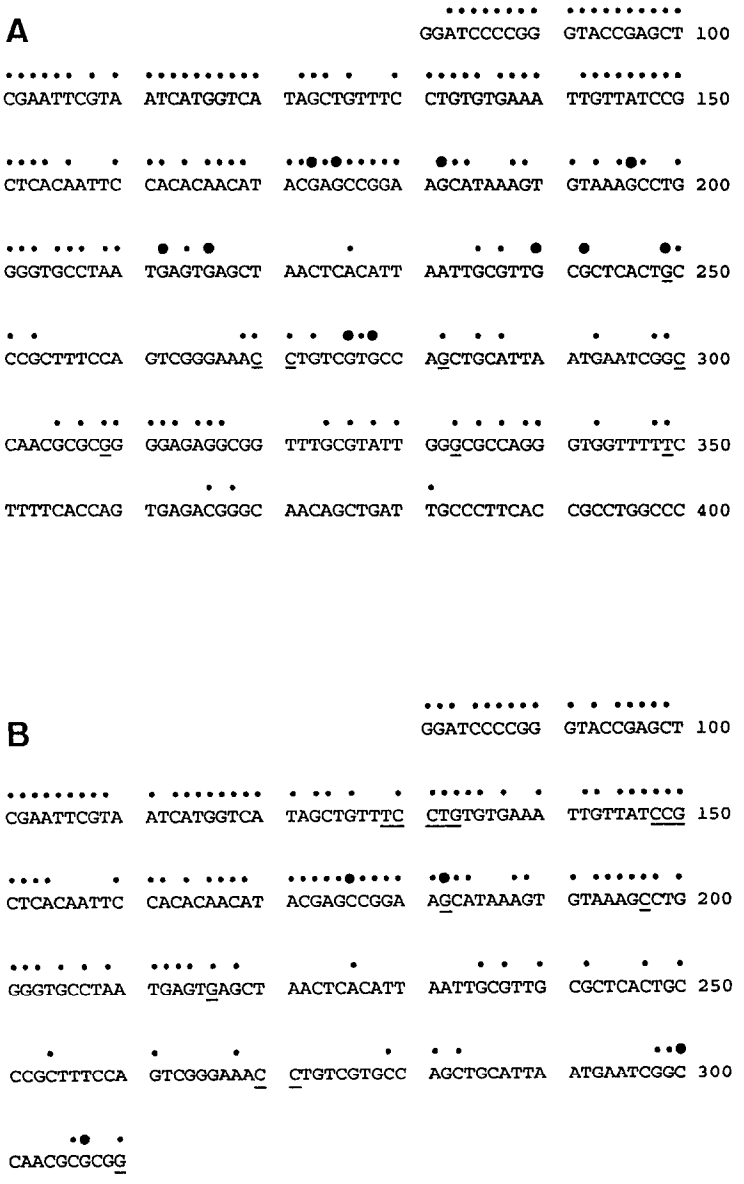


Fig. 6. Positions of base-specific inhibition of DNA polymerase action by the metal complexes in Figs. 3 and 5. (A) Cu(II)–SA complex; (B) Cu(II)–DEPD complex Key: (●) and (•) indicate the termination sites of the syntheses on DNA reacted with and without the complexes, respectively.

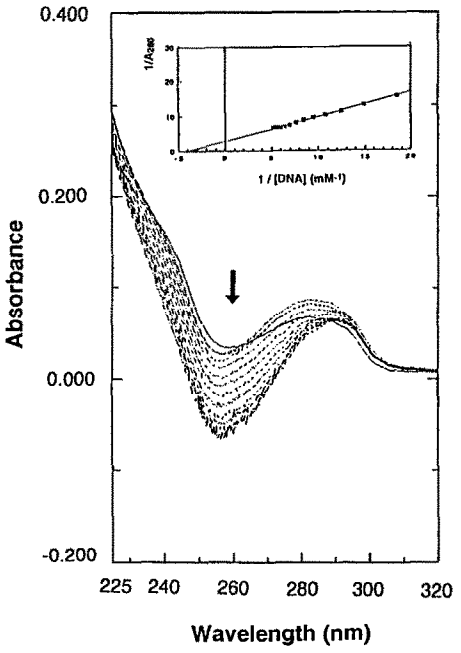


Fig. 7. Differential absorption spectral changes of Cu(II)–VP-16 complex upon titration of calf thymus DNA. A mixture of 15 μM Cu(II) and 15 μM VP-16 was titrated with fifteen 10-μL portions of 12.78 mM DNA. The arrow shows the decrease of absorbance near 260 nm with the addition of DNA. Inset: Lineweaver–Burk plot.

present study reveals further that the site-specific cleavages of DNA may be attributed to the affinity of these complexes for DNA. We are continuing our works to obtain evidence for binding regions of these complexes to DNA, e.g. by the combined use of DNase I footprinting and spectroscopic methods, to determine the relationship between base-specificity and cell cytotoxicity.

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