0026-895X/91/060965-09$03.00/0

MOLECULAR PHARMACOLOGY, 40:965-973



# Metal- and Photo-Induced Cleavage of DNA by Podophyllotoxin, Etoposide, and Their Related Compounds

HIROMU SAKURAI, TAKAO MIKI, YASUHIRO IMAKURA, MASAYUKI SHIBUYA, and KUO-HSIUNG LEE

*Kyoto Pharmaceutical University, Yamashina-ku, Kyoto 607, Japan (H.S.), Faculty of Pharmaceutical Sciences, University of Tokushima, Sho-machi 1, Tokushima 770, Japan (T.M., M.S.), Faculty of Sciences, Naruto University of Education, Takashima, Naruto-shi, Tokushima 772, Japan (Y.I.), and Natural Products Laboratory, Division of Medicinal Chemistry and Natural Products, School of Pharmacy, University of North Carolina, Chapel Hill, North Carolina 27599 (K.-H.L.)*

Received February 11, 1991; Accepted September 4, 1991

## SUMMARY

Podophyllotoxin (PD) and its derivative etoposide (VP-16), a clinically useful anticancer drug, exhibit different mechanisms of action. PD binds specifically to tubulin to prevent its polymerization, whereas VP-16 lacks this action. The DNA strand breakage caused by VP-16 is thought to be due to its interaction with topoisomerase II or to free radical formation by oxidation of its 4′-phenolic hydroxyl group to a semiquinone free radical. We have demonstrated that PD, VP-16, 4′-demethylepipodophyllotoxin (DEPD), and syringic acid (SA) exhibit no DNA-cleaving activity but, in the presence of metal ions such as $Cu^{2+}$ and $Fe^{3+}$, DEPD and SA form metal complexes, which in turn show high activity for DNA strand scission at pH 7.8 under air. Furthermore, it was found that DNA cleavage was greatly promoted by irradiation with UV light. The PD-$Fe^{3+}$ system at pH 7.8 showed very low DNA-cleaving activity, but irradiation with UV light in the system induced almost complete DNA breakage. DNA cleavages were significantly inhibited in the presence of hydroxyl radical scavengers, such as sodium benzoate and dimethylurea, in the $Cu^{2+}$-SA and $Fe^{3+}$-PD systems, with or without UV irradiation. These reactions were investigated by optical and ESR spectra, coupled with ESR spin-trapping techniques, by which the formation of hydroxy radicals was clearly detected in all systems. These findings have led us to a new proposal of the metal- and photo-induced mechanism for understanding the antitumor action of PD, VP-16, and their related compounds.

Recently, an extensive effort has been directed to the identification and characterization of molecules that mediate DNA strand scission. Antitumor natural products that cleave DNA have become the target of study because of their novel mechanism of action and for the development of new synthetic analogs. PD was isolated from the extracts of the roots of either *Podophyllum peltatum L.* or *Podophyllum emodi*, which have been used for centuries as cathartics and anthelmintics (1–3). Because PD was reported to inhibit the growth of cancer cells in animals, many researchers have investigated the antitumor activity of PD and its derivatives (Refs. 4 and 5 and references cited therein). PD inhibits microtubule assembly in the mitotic spindle apparatus by binding to the tubulin dimer (6). Unlike other compounds, such as vinblastine and vincristine, that exhibit a similar mechanism of action, PD itself has little potential as an antitumor drug, due to its poor solubility (6). However, attempts to search for useful analogs of PD have led to the discovery of VP-16 (etoposide) and VM-26 (teniposide) (1–3). These two analogs are antitumor drugs currently in use for the treatment of a number of human cancers, including testicular and small cell lung cancers and lymphoma (7, 8). They are also used clinically in combination with other antitumor drugs, such as Adriamycin, cisplatin, and cyclophosphamide (9, 10). In contrast to PD, VP-16 does not bind to tubulin and does not inhibit microtubule assembly. VP-16 induces both single- and double-stranded DNA scissions in several types of tumor cells. Loike and Horwitz (11) have found that VP-16 causes single-stranded breaks in DNA in HeLa cells. Wozniak and Ross (12) have presented evidence that the mechanism of cytotoxicity of VP-16 in L-1210 cells may involve DNA strand cleavage. Furthermore, it has been reported that in the presence of several enzymatic systems, such as cytochrome P-450/NADPH, horseradish peroxidase/$H_2O_2$, and prostaglandin synthetase/arachidonic acid, VP-16 undergoes *O*-demethylation to yield reactive metabolites capable of irreversible binding to cellular macromolecules (13–19). These reactive intermediates, such as catechol or quinone derivatives, were shown to produce active oxygen species in the presence of Fe(III)-EDTA (20).

This work was supported by grants from the Ministry of Education, Science, and Culture of Japan (03241220) (H.S.) and from the American Cancer Society (CH-370D) (K.-H.L.). This work was presented in part at The 1989 International Chemical Congress of Pacific Basin Societies, Honolulu, Hawaii, December 17–22, 1989 (Paper 154).

**ABBREVIATIONS:** PD, podophyllotoxin; DEPD, 4′-demethylepipodophyllotoxin; SA, syringic acid; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; BLM, bleomycin; DMSO, dimethyl sulfoxide; DMF, *N,N*-dimethylformamide; DABCO, 1,4-diazabicyclo[2,2,2]octane.

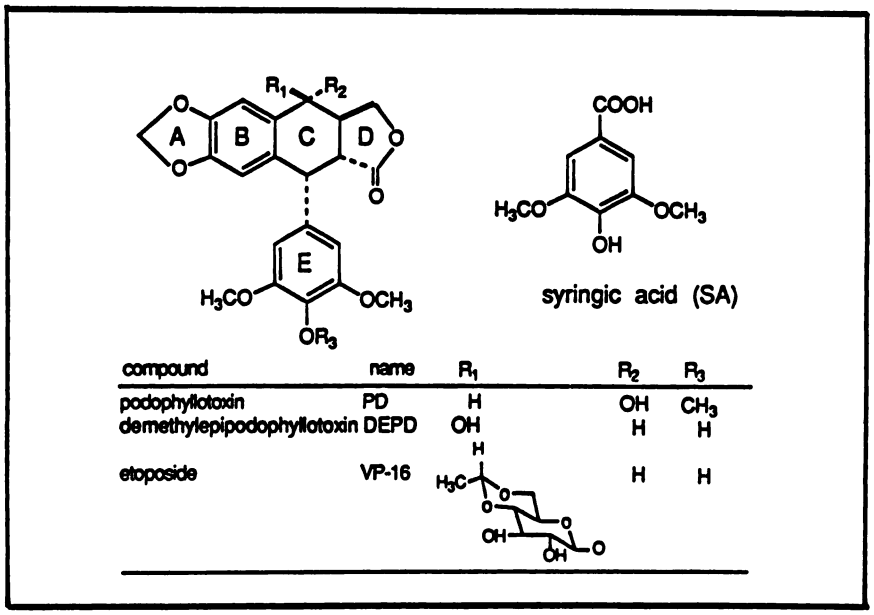


**Fig. 1.** Structures of PD, DEPD, VP-16, and SA.

TABLE 1

**DNA-cleaving activity of PD-related compounds in the presence of various metal ions at pH 7.8 and under UV irradiation**

Concentrations of the compounds and metal ions were 0.1 mM.

| System | Compound | UV irradiation | DNA-cleaving activity | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | $Fe^{3+}$ | $Cu^{2+}$ | $VO^{2+}$ | $Mn^{2+}$ | $Zn^{2+}$ | $Mg^{2+}$ | $Ni^{2+}$ | $Ca^{2+}$ | None |
| | | | % | | | | | | | | |
| a | SA | No | 69 | 34 | 24 | 7 | 10 | 10 | 5 | 9 | 4 |
| b | SA | Yes | 94 | 94 | 9 | 6 | 2 | 5 | 38 | 0 | 2 |
| c | DEPD | No | 82 | 86 | 15 | 17 | 7 | 14 | 15 | 10 | 7 |
| d | VP-16 | No | 19 | 24 | 20 | 16 | 14 | 10 | 12 | 7 | 3 |
| e | PD | No | 15 | 10 | 14 | 11 | 12 | 13 | 12 | 9 | 5 |
| f | PD | Yes | 95 | 18 | 12 | 4 | 4 | 2 | 2 | 2 | 2 |

TABLE 2

**Metal ion-dependent DNA cleavage of SA or DEPD at pH 7.8 and under UV irradiation**

| System | Compound | UV irradiation | Ratio of [compound]/[metal ion] | DNA-cleaving activity | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | $Cu^{2+}$ | | | | | | | $Fe^{3+}$ | | | | | | |
| | | | | 0[a] | 10 | 20 | 50 | 100 | 200 | 400 | 0[a] | 10 | 20 | 50 | 100 | 200 | 400 |
| | | | | % | | | | | | | | | | | | | |
| a | SA | No | 25 | 5 | 19 | 23 | 30 | 86 | 92 | 92 | 8 | 14 | 15 | 36 | 84 | 90 | 90 |
| b | SA | Yes | 20 | 7 | 12 | 27 | 66 | 94 | 94 | 94 | 4 | 16 | 16 | 54 | 93 | 93 | 93 |
| c | DEPD | No | 10 | 6 | 12 | 15 | 21 | 90 | 90 | 90 | 9 | 9 | 22 | 19 | 88 | 88 | 88 |
| d | DEPD | Yes | 10 | 3 | 14 | 16 | 33 | 93 | 93 | 93 | 7 | 19 | 20 | 24 | 89 | 89 | 89 |

[a] μM.

The precise mechanism of DNA breakage induced by VP-16 is still not known, but it is thought to be due to drug stabilization of a cleavable complex formed between DNA topoisomerase II and DNA (21–23).

While investigating the mechanism of DNA cleavage by chemical compounds (24–26), we have found that PD and its related compounds (Fig. 1), including VP-16, DEPD, and SA, form complexes with several metal ions. These metal complexes show high activity for DNA strand breakage at pH 7.8 under air. Furthermore, this DNA cleavage was shown to be greatly promoted by irradiation with UV light. On the basis of these findings, we report in this paper a new mechanism of antitumor action of PD, VP-16, and their related compounds.

## Experimental Procedures

**Materials.** PD was isolated from the ethanol extract of the roots and rhizomes of *Podophyllum hexandrum* (Atomic Chemetals Corp., Farmingdale, NY), together with α-peltatin, desoxypodophyllotoxin, and 4′-demethylpodophyllotoxin, by silica gel chromatography with the solvent system of $CHCl_3$/acetone (9:1, 7:1, and 5:1), according to the modified method of Thurston *et al.* (27, 28). DEPD was prepared by introduction of hydrogen bromide gas into a mixture of PD and anhydrous $CH_2Cl_2$ at 0°, as described previously (26). VP-16 was purified by silica gel column chromatography from a product (Vepesid) of Bristol-Myers Company (Wallingford, CT), and SA was from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Plasmid Coe E1 DNA (>90% supercoiled form) was obtained from Nippon Gene Co. (Tokyo, Japan). Special pure grade DMPO, purified by distillation, was purchased from Labotec Co. (Tokyo, Japan) and used without further purification. Superoxide dismutase from bovine erythrocytes and catalase from bovine liver were purchased from Sigma Chemical Co. (St. Louis, MO). Ferric ammonium sulfate, cupric chloride, EDTA, agarose S, Tris, formic acid, DMSO, ethanol, DMF, sodium benzoate, 1,1-dimethylurea, ethidium bromide, and DABCO were obtained from Wako Pure Chem-

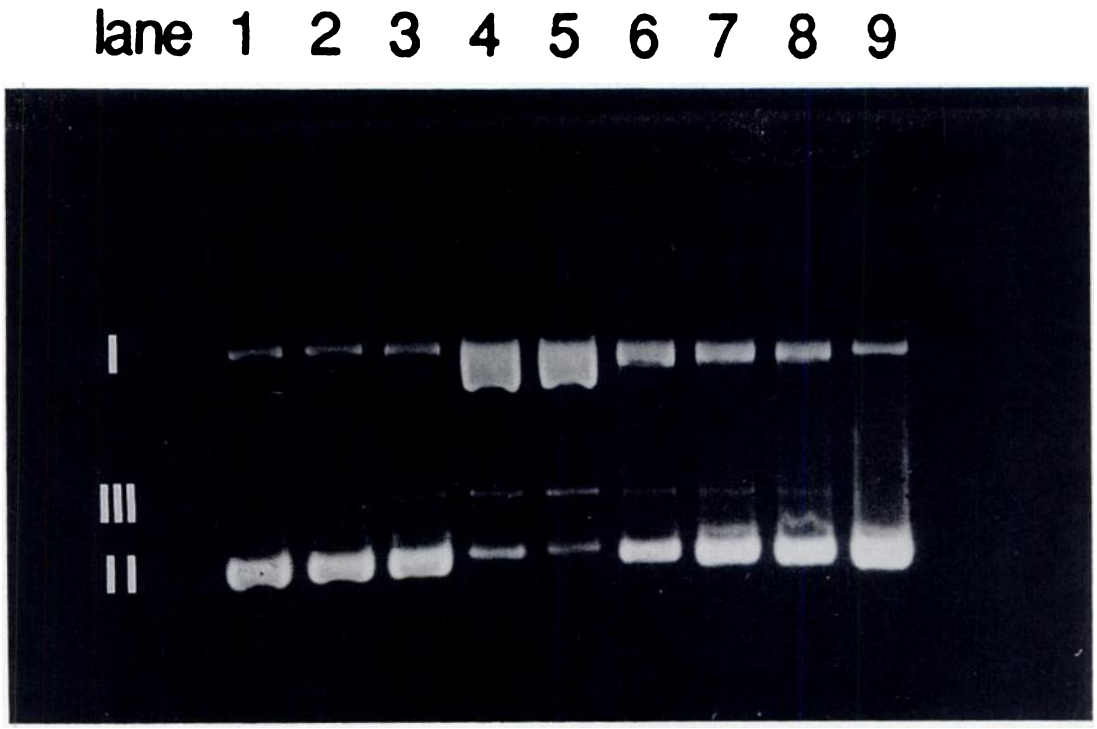


**Fig. 2.** Effects of SA concentration on DNA cleavage in the presence of $Cu^{2+}$ ion. Col E1 DNA (0.3 μg) was incubated with 0.1 mM $Cu^{2+}$ and various concentrations of SA under air for 60 min at 37°. *Lane 1*, DNA alone; *lane 2*, DNA plus 40 mM SA; *lane 3*, DNA plus 0.1 mM $Cu^{2+}$; *lanes 4–9*, DNA plus 0.1 mM $Cu^{2+}$ plus 2.5, 5.0, 10.0, 20.0, 25.0, and 40.0 mM SA, respectively.

TABLE 3

**Optimal molar ratio of the compound to metal ion for DNA cleavage by $Cu^{2+}$ or $Fe^{3+}$ complexes of PD-related compounds**

Concentration of metal ions was 0.1 mM (constant). The values in parenthesis show the [compound]/[metal] ratio at which the maximum DNA-cleaving activity was observed.

| Complexes | DNA-cleaving activity | | | |
|---|---|---|---|---|
| | pH 7.8 | | pH 5.0 | |
| | No irradiation | UV irradiation | No irradiation | UV irradiation |
| | % | | | |
| $Cu^{2+}$-SA | 94 (25–50) | 100 (>10)[a] | 100 (>10) | |
| $Cu^{2+}$-DEPD | 90 (>25)[b] | 100 (>25)[c] | 97 (>5) | |
| $Cu^{2+}$-VP-16 | 39 (>10) | 50 (10–100) | 30 (>100) | 33 (>25) |
| $Cu^{2+}$-PD | 7 (10–15) | 14 (10–50) | 30 (>200) | 20 (10–50) |
| $Fe^{3+}$-SA | 93 (>10) | 94 (>50)[d] | 98 (>10) | |
| $Fe^{3+}$-DEPD | 95 (>5) | 96 (>1) | 94 (>15) | |
| $Fe^{3+}$-VP-16 | 56 (15–50) | 85 (3) | 88 (3) | 83 (5) |
| $Fe^{3+}$-PD | 30 (>20) | 90 (>10) | 60 (10–25) | 88 (>50) |

[a–d] Formation of form III DNA was observed in these systems at 45%, 31%, 53%, and 16%, respectively.

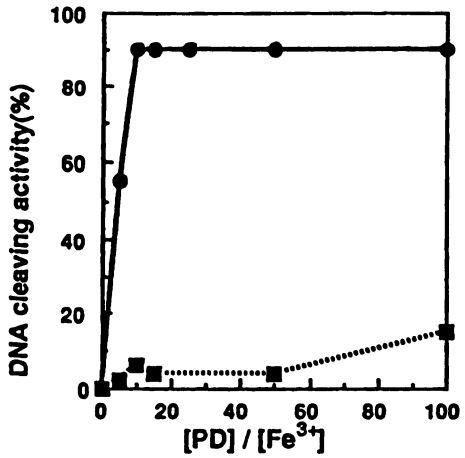


**Fig. 3.** DNA cleavage by $Fe^{3+}$-PD complexes at pH 7.8. The concentrations of $Fe^{3+}$ and PD were 0.1 mM and 0.1–10 mM, respectively. ●, UV irradiation; ■, no irradiation.

ical Industries (Osaka, Japan). Other materials were of special reagent grade and were used without further purification. The solutions of metal ions were standardized complexometrically with EDTA.

**DNA-cleaving activity.** Supercoiled plasmid DNA (0.2–0.3 μg) was incubated in 10 μl of a reaction mixture containing various concentrations of metal ions (10–400 μM) and VP-16, DEPD, PD, or SA (0.1–25 mM), in 20 mM Tris-acetate buffer, pH 7.8 or pH 5.0, with or without irradiation at >250 nm (30 mW) at 37°. Stock solutions of all ligands were prepared in a mixed solvent of acetonitrile/Tris-acetate buffer (4:1). Reactions were started by addition of the ligand and stopped after 60 min by 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. The samples were loaded on a 1% neutral agarose gel containing 20 mM Tris-acetate buffer and were subjected to electrophoresis in a horizontal slab gel apparatus (50 V for 8–10 hr). The gel was stained with a solution of 1 μg/ml ethidium bromide for 20–30 min. Bands of DNA were detected and photographed (Polaroid 600 SE camera with an orange filter) under UV light (253.7 nm) in a dark room. Determination of form I (supercoiled form), form II (nicked form), and form III (linear form) was performed with a dual-wavelength flying spot scanner (Shimadzu CS-9000, Kyoto, Japan). DNA-cleaving activity of the complex was estimated by the following equation:

$$\text{DNA-cleaving activity (\%)} = \frac{(\text{forms II + III})_{\text{complex}} - (\text{forms II + III})_{\text{ligand}}}{(\text{forms I + II + III})_{\text{ligand}}} \times 100$$

**Complex formation.** Complex formations between the ligands and metal ions were determined by the continuous-variation method, at various pH values (pH 4–8). The electronic absorption spectra were measured with a JASCO U-best 50 UV/visible spectrometer (Tokyo, Japan), at room temperature. The ESR spectra were recorded with a JEOL FEIXG ESR spectrometer (Tokyo, Japan) at 295°K or 77°K, with a modulation frequency of 100 KHz, modulation amplitude of 6.3

TABLE 4

**Inhibitory effects of active oxygen scavengers on DNA cleavage by $Cu^{2+}$-SA and $Fe^{3+}$-PD complexes at pH 7.8**

Concentrations of metal ions and the compounds were 0.1 mM and 2.0 mM, respectively, in all systems. $IC_{50}$ represents the concentration producing 50% inhibition of DNA cleavage in the presence of an active oxygen scavenger.

| Active oxygen scavengers | Concentration | $Cu^{2+}$-SA complex | | | | $Fe^{3+}$-PD complex, UV irradiation | |
|---|---|---|---|---|---|---|---|
| | | No irradiation | | UV irradiation | | | |
| | | Inhibition | $IC_{50}$ | Inhibition | $IC_{50}$ | Inhibition | $IC_{50}$ |
| | | % | mM | % | mM | % | mM |
| Sodium benzoate | 20 mM | 98 | 1.3 | 100 | 1.0 | 90 | 1.9 |
| 1,1-Dimethylurea | 20 mM | 66 | 1.5 | 70 | 1.4 | 65 | 1.6 |
| Formic acid | 20 mM | 64 | 2.3 | 72 | 2.1 | 69 | 2.9 |
| DMSO | 20 mM | 74 | 3.4 | 75 | 3.0 | 76 | 3.5 |
| Ethanol | 20 mM | 87 | 5.4 | 93 | 5.0 | 82 | 2.9 |
| DMF | 20 mM | 71 | 9.7 | 74 | 9.5 | 66 | 2.9 |
| Superoxide dismutase | 5–100 units/ml | 0 | | | | 6 | |
| Catalase | 10–100 units/ml | 2 | | | | 8 | |
| DABCO | 0.1–200 mM | 2 | | | | 5 | |

TABLE 5

**Molecular dioxygen consumption by metal-SA complexes at pH 7.8**

The solutions were prepared in 20 mM Tris-acetate buffer, pH 7.8. The concentrations of metal ions and SA were 0.1 mM and 2 mM, respectively.

| System | Oxygen consumption |
|---|---|
| | nmol/min |
| $Cu^{2+}$ or $Fe^{3+}$ | 0 |
| SA | 0 |
| $Cu^{2+}$-SA | 206 ± 27 |
| $Fe^{3+}$-SA | 80 ± 73 |

G, and microwave power of 5 mW. The spectrometer was calibrated with a Takeda Riken TR 5212 frequency counter (Osaka, Japan). Tetracyanoquinodimethane radical-lithium salt ($g$ = 2.00252) and Mn(II) doped in MgO were used as the standards.

**ESR spin-trapping method.** A mixture (100 μl) of metal ions (0.1 mM), ligand (2 mM), and DMPO (0.4 M), in 20 mM Tris-acetate buffer (pH 7.8 or pH 5.0), was transferred into a flat quartz ESR cuvette (5 mm phi and 0.3-mm thickness), which was fixed to the cavity of the ESR spectrometer. Recording of the ESR spectra was started 30 sec after the mixing of DMPO at 22°, and each scan took 2 min. Instrument settings were as follows: magnetic field, 3350 ± 50 gauss; microwave power, 5 mW; field modulation frequency, 100 KHz; modulation amplitude, 1 G; amplitude, 2000 G; and response, 0.3 sec. The ESR spectra of DMPO-OH adducts were identified by the hyperfine parameters and computer simulation.

**Oxygen consumption by the complex.** Oxygen concentrations in solution were measured with a biological oxygen monitor (model 5331; Yellow Springs Instruments, Yellow Springs, OH), which was attached to a Hitachi recorder. The oxygen vessel was surrounded by circulating water maintained, with a thermostat with a temperature control, at 37 ± 0.1°. Stirring was maintained with a magnetic stirrer. The final volume of the vessel was 2.8 ml. The reaction was followed for at least 30 min. The calculation of oxygen consumption was based on the presence of 250 μM oxygen in air-equilibrated buffer and on 2.8 ml of reaction volume.

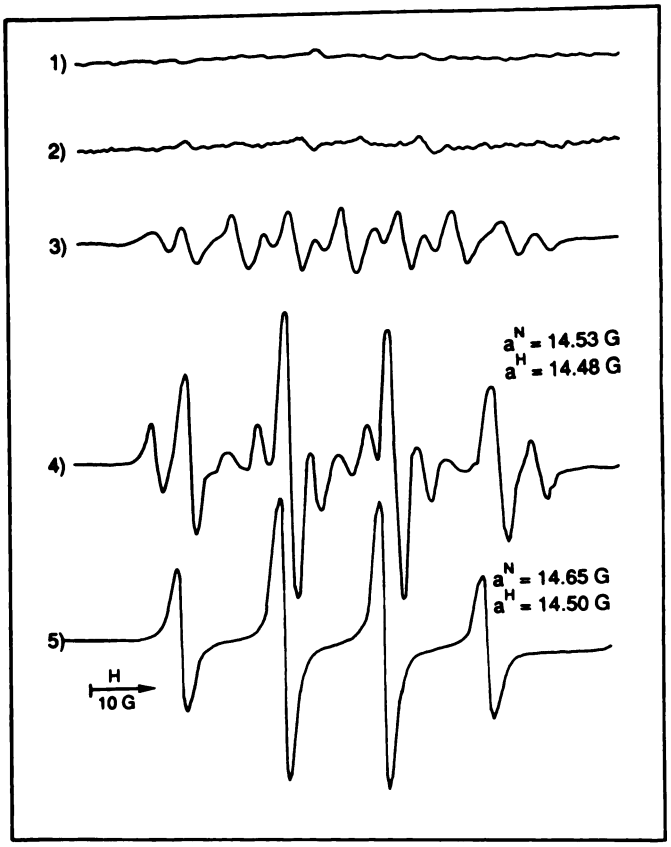


**Fig. 4.** Spin-trapping of hydroxyl radicals in the $Cu^{2+}$-SA complex system and its computer simulation. The solutions were prepared in 20 mM Tris-acetate buffer at pH 7.8. After the addition of DMPO (0.4 M), the ESR spectra were recorded within 30 sec. The concentrations of SA and $Cu^{2+}$ were 2 mM and 0.1 mM, respectively. *Trace 1*, SA alone; *trace 2*, $Cu^{2+}$ alone; *trace 3*, SA plus $Cu^{2+}$; *trace 4*, SA plus $Cu^{2+}$ under UV irradiation; *trace 5*, computer-simulated spectrum of DMPO-OH adduct.

## Results

**Metal ion-induced cleavage of DNA by PD-related compounds.** The DNA-cleaving activities of the metal complexes of PD and its related compounds, such as SA, DEPD, and VP-16 (Fig. 1), were monitored by horizontal agarose gel electrophoresis. Essentially no DNA cleavage was observed with either PD, its related compounds, or metal ions alone, at the concentrations tested in the present study. DNA strand scissions were induced by PD-related compounds in the presence of various types of metal ions at pH 7.8 (Table 1, systems a, c, d, and e). The combinations of DEPD with $Fe^{3+}$ or $Cu^{2+}$ showed the highest degree of DNA strand scission, which was followed by the systems containing SA. The addition of <0.1 mM $Cu^{2+}$ or $Fe^{3+}$ did not stimulate DNA cleavage (Table 2, systems a and c). DNA-cleaving activity depended on the ratio of the compound to the metal ion concentrations. For example, in the system of SA-$Cu^{2+}$, DNA strand scission reached a

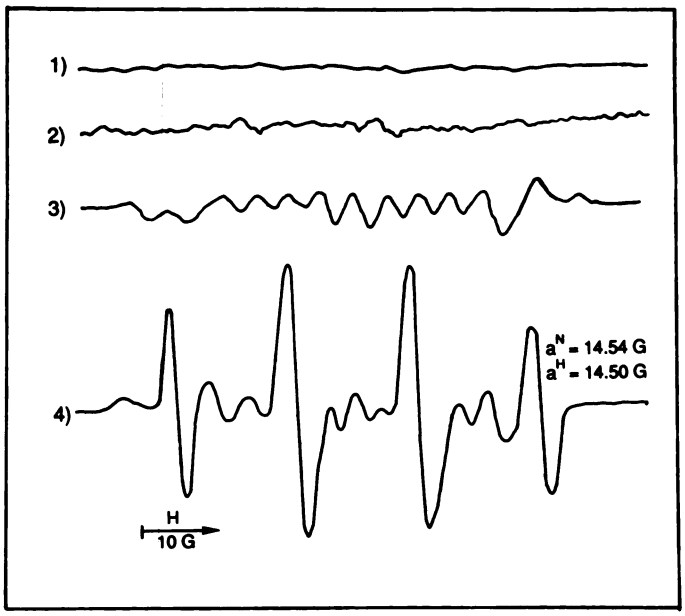


**Fig. 5.** Spin-trapping of hydroxyl radicals in the $Fe^{3+}$-PD complex system. The solutions were prepared in 20 mM Tris-acetate buffer at pH 7.8, and ESR spectra were recorded as for Fig. 4. The concentrations of PD and $Fe^{3+}$ were 2 mM and 0.1 mM, respectively. *Trace 1*, PD alone; *trace 2*, $Fe^{3+}$ alone; *trace 3*, PD plus $Fe^{3+}$; *trace 4*, PD plus $Fe^{3+}$ under UV irradiation.

maximum value at a ratio of SA to $Cu^{2+}$ of 25–50:1, using a $Cu^{2+}$ ion concentration of 0.1 mM, and excessive addition of SA to $Cu^{2+}$ over the ratio of SA to $Cu^{2+}$ of 50:1 inhibited DNA cleavage (Fig. 2), probably due to the stability of the complexes formed at these ratios. The optimal ratios of the compound to metal ion were also determined in other systems with a 0.1 mM metal ion concentration (Table 3). In general, DNA strand scissions were found to occur when the concentration of the metal ion was less than that of the ligand.

**Metal- and photo-induced cleavage of DNA by PD-related compounds.** In addition to the metal ions necessary for the exhibition of DNA-cleaving activity by PD-related compounds, the effect of UV light on the systems containing SA, PD, and DEPD was studied. As seen in Tables 1–3, the effects of UV irradiation in the presence of $Fe^{3+}$ or $Cu^{2+}$ were clearly observed. Other metal ions, however, did not promote DNA breakage. A remarkable enhancement of the DNA strand scission by PD was observed in the presence of $Fe^{3+}$ at pH 7.8. In the absence of PD in the system, DNA strand scission was not observed. Almost complete DNA strand scission occurred when the molar ratio of PD to $Fe^{3+}$ was 10:1, at the $Fe^{3+}$ concentration of 0.1 mM (Fig. 3 and Table 3). A similar effect was also observed at pH 5.0 (Table 3). In other systems containing $Cu^{2+}$ or $Fe^{3+}$, the effect of UV irradiation was found to be less significant. It is interesting to note that the double-strand scission, in terms of the formation of form III DNA, occurred in the systems containing SA and DEPD (data not shown).

**Involvement of active oxygen species in DNA cleavage by metals and PD-related compounds, with or without UV irradiation.** VP-16 possesses a dimethoxyphenol ring (E-ring) (Fig. 1) and shows reducing ability in cells. It has been demonstrated that VP-16 forms a metal complex in the presence of a metal ion, which would activate the molecular dioxygen under air. Therefore, it appears likely that the mechanism of the antitumor action of this compound may involve the formation of free radicals, such as active oxygen species. The free radicals participate in the degradation of the DNA molecule. In order to test this hypothesis, we examined DNA cleavage in the presence of several types of active oxygen scavengers in the systems $Cu^{2+}$-SA and $Fe^{3+}$-PD, with and without UV irradiation (Table 4). In the three systems examined, DNA strand scission was inhibited by hydroxyl radical scavengers, including sodium benzoate, 1,1-dimethylurea, formic acid, DMSO, ethanol, and DMF, and their $IC_{50}$ values were in the range of 1–10 mM. However, a superoxide anion scavenger (superoxide dismutase), hydrogen peroxide scavenger (catalase), and singlet oxygen quencher (DABCO) showed essentially no inhibitory effect.

Both $Cu^{2+}$- and $Fe^{3+}$-SA systems showed rapid dioxygen consumption at pH 7.8, whereas the metal ions or SA alone consumed no dioxygen under the same conditions (Table 5). The results described above clearly suggest the involvement of ·OH (derived from molecular dioxygen) in DNA cleavage by the metal-PD-related compounds. We attempted to detect the formation of ·OH in these systems by ESR experiments, using DMPO as the spin-trapping agent (Fig. 4). Although SA or $Cu^{2+}$ alone gave no signals, the $Cu^{2+}$-SA system displayed complicated ESR signals due to the formation of a DMPO-OH adduct, together with unidentified small signals. Under UV irradiation, a very intense signal due to a DMPO-OH adduct appeared ($a^N$ = 14.5 gauss; $a^H$ = 14.5 gauss), which was identical to the computer-simulated signal of the DMPO-OH adduct ($a^N$ = 14.7 gauss; $a^H$ = 14.5 gauss). Furthermore, the DMPO-OH signals were found to be greatly enhanced under UV irradiation in the $Fe^{3+}$-PD system (Fig. 5). In the absence of the ligands in these systems, enhancement of the ESR signals due to the DMPO-OH adduct was not observed. These observations of the increased formation of ·OH under UV irradiation corre-

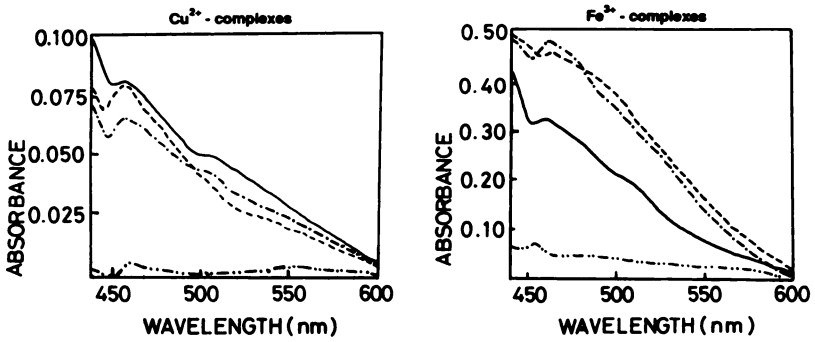


**Fig. 6.** Electronic absorption spectra of $Fe^{3+}$ or $Cu^{2+}$ complexes of PD-related compounds at pH 5.0. The solutions were prepared in 20 mM Tris-acetate buffer at pH 5.0. The concentrations of the compounds and metal ions were both 1.67 mM. ——, SA; –·–·–, PD; – – –, DEPD; –·–, VP-16.

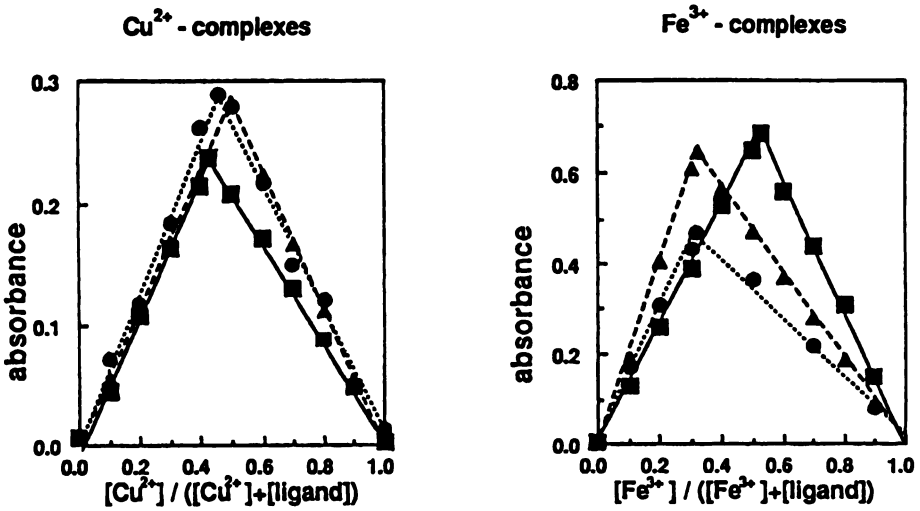


**Fig. 7.** Determination of complex formation ratio by continuous-variation method. ▲, DEPD; ●, SA; ■, VP-16. In both $Cu^{2+}$ and $Fe^{3+}$ ligand systems at pH 5.0, the absorbance at 460 nm due to the complex formations was monitored.

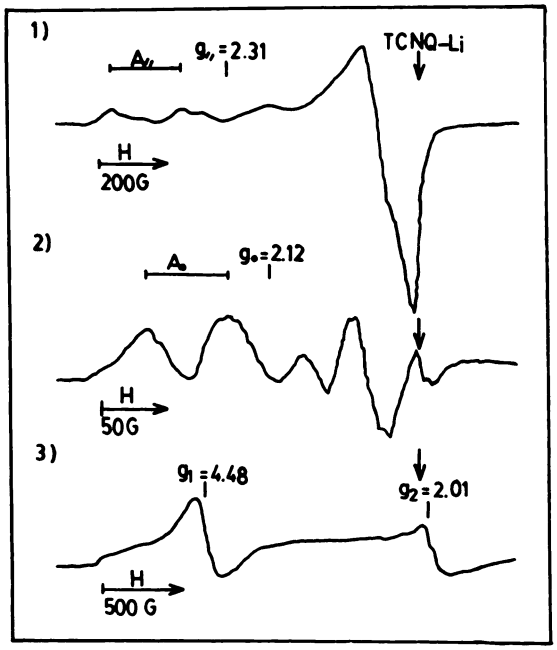


**Fig. 8.** ESR spectra of $Cu^{2+}$- or $Fe^{3+}$-SA complexes at pH 5.0. The solutions were prepared in 20 mM Tris-acetate buffer at pH 5.0. The concentrations of $Cu^{2+}$ and SA were both 0.5 mM for *traces 1* and *2*, and those of $Fe^{3+}$ and SA were both 1.6 mM for *trace 3*. The spectra for *traces 1* and *3* were recorded at 77°K and that for *trace 2* was at 22°. *TCNQ-Li*, tetracyanoquinodimethane radical lithium salt.

spond well to the results on DNA cleavage obtained in SA-$Cu^{2+}$ and PD-$Fe^{3+}$ systems under UV irradiation. In the SA-$Cu^{2+}$ system, 94% of DNA was degraded by UV irradiation, whereas 34% DNA breakage was observed without UV light (Table 1, systems a and b). In the PD-$Fe^{3+}$ system, UV irradiation induced 95% DNA cleavage, but only 15% of DNA was nicked without UV light (Table 1, systems e and f). The experimental results described above strongly indicated the involvement of ·OH in the DNA-cleaving activities induced by the systems with these metal complexes.

**Metal complex formation of PD-related compounds.** Our previous results on the metal ion-induced cleavage of DNA by PD-related compounds indicated that the formation of complexes between PD-related compounds and the metal ions may be involved in their DNA-cleaving action. We, therefore, attempted to demonstrate whether the complexing of PD-related compounds with the metal ions actually took place under the conditions examined earlier. Because the solutions prepared at pH 7.8 inevitably caused turbidity, their electronic absorption spectra were not recorded. The electronic absorption spectra in the visible region at pH 5.0 are shown in Fig. 6. Compounds that contained a free phenol group, such as SA, DEPD, and VP-16, showed absorption maxima at 460 nm and 510 nm (shoulder). PD, which has three methoxy groups (no hydroxy group on ring E), gave no apparent absorption in this spectral region. Continuous-variation methods monitored at about 460 nm (pH 5.0) indicated that (i) approximately a 1:1 ligand-$Cu^{2+}$ complex was formed in the case of SA, DEPD, and VP-16, (ii) a 2:1 ligand-$Fe^{3+}$ complex was formed in the systems containing SA and DEPD, and (iii) approximately a 1:1 VP-16/$Fe^{3+}$ complex was formed (Fig. 7).

Typical ESR spectra of the $Cu^{2+}$- or $Fe^{3+}$-SA complexes are shown in Fig. 8. The ESR and bonding parameters estimated for $Cu^{2+}$ and $Fe^{3+}$ complexes, together with other types of complexes, are summarized in Tables 6 and 7. These data clearly indicate that SA, DEPD, and VP-16 bind with $Cu^{2+}$ through four oxygen ligands to form the $CuO_4$ coordination structure. The bonding parameters $\alpha^2$ and $\beta^2$, calculated on the basis of molecular orbital theory (29–33) for the complexes of PD-related compounds, show values similar to those calculated for $Cu^{2+}$-oxalate or $Cu^{2+}$-citrate complex. These values suggested that the complexing of SA or DEPD with $Cu^{2+}$ consists of a moderately covalent $\sigma$ bonding, with an $\alpha^2$ value of 0.8, and a very weak out-of-plane $\pi$ bonding, with a $\beta^2$ value of about 1.0. The complexing of VP-16 with $Cu^{2+}$ is of more covalent nature. The $Fe^{3+}$ complexes gave a $g$ value of 4.3. This is typical of those of the non-heme iron complexes (34), which show a tetrahedral coordination mode.

## Discussion

It has been shown that VP-16 induces DNA strand breaks in HeLa cells and L-1210 cells (11, 12), and its cytotoxicity may be due to this DNA damage. It has also been suggested that VP-16 exerts its anticancer effect by poisoning type II topoisomerase without binding to DNA (21). Recent reports have shown that VP-16 undergoes *O*-demethylation when ac-

TABLE 6

**ESR parameters of $Cu^{2+}$ complexes of PD-related compounds**

ESR spectra were recorded at 77°K and 22°.

| $Cu^{2+}$ complexes | $g_{//}$ | $g_0$ | $g_\perp$ | $\lvert A_{//}\rvert$ ($10^{-4}$) | $\lvert A_0\rvert$ ($10^{-4}$) | $\lvert A_\perp\rvert$ ($10^{-4}$) | $\alpha^2$ | $\beta^2$ |
|---|---|---|---|---|---|---|---|---|
| | | | | $cm^{-1}$ | $cm^{-1}$ | $cm^{-1}$ | | |
| SA | 2.31 | 2.12 | 2.03 | 162 | 76 | 29 | 0.81 | 0.97 |
| DEPD | 2.31 | 2.11 | 2.01 | 161 | 72 | 28 | 0.79 | 0.98 |
| VP-16 | 2.27 | 2.11 | 2.04 | 164 | 74 | 29 | 0.78 | 0.88 |
| Oxalate[a] | 2.32 | 2.16 | 2.08 | 170 | | | 0.84 | 0.92 |
| Citrate[a] | 2.35 | 2.16 | 2.07 | 150 | | | 0.82 | 0.93 |
| Acetyl-acetonate[b] | 2.27 | 2.12 | 2.05 | 160 | 67 | 20 | 0.75 | 0.87 |
| Bissalicylaldoxime[c] | 2.17 | 2.07 | 2.02 | 183 | 88 | 41 | 0.76 | 0.79 |
| Bissalicylaldimine[c] | 2.14 | 2.10 | 2.08 | 168 | 67 | 16 | 0.69 | 0.64 |
| Diethyldithiocarbamate[c] | 2.10 | 2.06 | 2.04 | 154 | 78 | 40 | 0.59 | 0.55 |
| EDTA[a] | 2.34 | 2.17 | 2.09 | 160 | | | 0.84 | 0.89 |
| Imidazole[a] | 2.27 | 2.13 | 2.06 | 180 | | | 0.82 | 0.89 |
| Histidine[a] | 2.23 | 2.12 | 2.06 | 180 | | | 0.78 | 0.78 |

[a] From Ref. 31.
[b] From Refs. 31 and 32.
[c] From Ref. 33.

TABLE 7

**ESR parameters of $Fe^{3+}$ complexes of PD-related compounds**

ESR spectra were recorded at 77°K.

| $Fe^{3+}$ complexes | $g_1$ | $g_2$ |
|---|---|---|
| SA | 4.48 | 2.01 |
| PD | 4.47 | 2.01 |
| DEPD | 4.47 | 2.01 |
| VP-16 | 4.43 | 2.01 |
| Rubredoxin[a] | 4.30 | |

[a] From Ref. 32.

tivated by peroxidases, and this *O*-demethylation may be an important mechanism for the formation of reactive intermediates, such as a 3′,4′-hydroxy derivative, and may play a role in the mechanism of action of VP-16 (13–15). We have demonstrated that the PD-related compounds themselves exhibit no DNA-cleaving activity but in the presence of metal ions, such as $Cu^{2+}$ and $Fe^{3+}$, they induce DNA strand scission. Furthermore, this DNA cleavage was shown to be greatly promoted by UV irradiation (Tables 1, 2, and 3), suggesting that it may involve the *O*-demethylation of metal complexes.[1]

Recently, the role that active oxygen species play in the field of pharmacology, as well as life sciences, has received a great deal of attention. It has become important especially in understanding the mechanism of anticancer agents (35, 36). The cytotoxicity of PD or VP-16 may also be related to the formation of active oxygen species. Based upon our studies on the effect of active oxygen scavengers on DNA cleavage by metal complexes of PD-related compounds, as well as the ESR spin-trapping experiments, it was shown that the hydroxy radical ·OH (but not $O_2^-$, $H_2O_2$, or $^1O_2$) generated from molecular dioxygen is responsible for the metal- and photo-induced DNA

[1] The details of the structures and stereochemistries of these compounds, as well as the mechanism for the DNA-cleaving activity of the PD-$Fe^{3+}$ systems under UV irradiation, will be reported elsewhere.

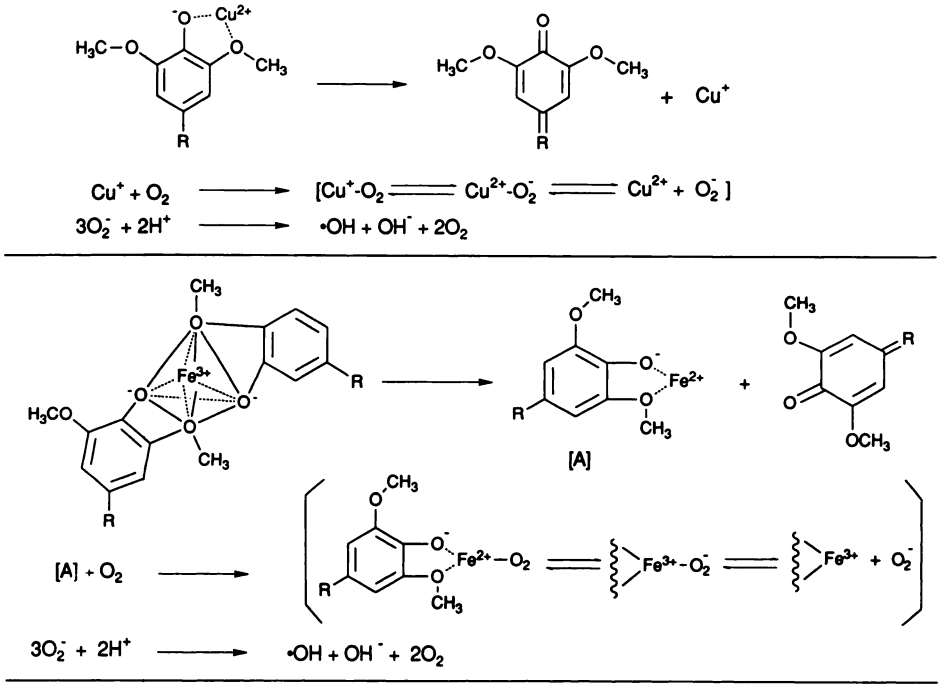


**Fig. 9.** Proposed mechanism for DNA-cleaving activities of the metal complexes.

breaks produced by the PD-related compounds (Table 4 and Figs. 4 and 5). We expect that the ·OH generation might be involved in these reactions. Such ·OH formation has already been observed in the dihydroxy VP-16/$Fe^{3+}$ system in the presence of hydrogen peroxide (37). Addition of mannitol, which acts as a ·OH scavenger, inhibited DNA nicking, but superoxide dismutase had no effect (38), which is similar to our present results. It appears likely that PD-related compounds may form complexes with metal ions, such as $Cu^{2+}$ or $Fe^{3+}$, to generate an active oxygen species that is responsible for DNA strand cleavage. BLM has been shown to complex with metals to cause DNA strand scission in the presence of dioxygen. $Fe^{3+}$ binds to four nitrogen atoms in the molecule of BLM to form a square planar $FeN_4$ coordination structure. The axial positions are occupied by a nitrogen in the BLM molecule and a superoxide anion (39–42). In PD-related compounds, $Cu^{2+}$ forms 1:1 $Cu^{2+}$-ligand complexes. $Fe^{3+}$ also forms 1:1 $Fe^{3+}$-VP-16 complexes, but it binds to SA and DEPD in the ratio of 1:2. The ESR spectra showed that $Cu^{2+}$ complexes possess a $CuO_4$ coordination structure. This suggests that two oxygen ligands are phenol and phenoxy groups, and the others come from the two oxygens of coordinate water molecules. The bonding parameters indicated that the complexing of VP-16 with $Cu^{2+}$ is of more covalent nature than those of SA and DEPD. The $Fe^{3+}$ complexes possess a tetrahedral structure of the non-heme iron type.

The degree of DNA-cleaving properties was found to vary, depending upon the complexes employed. SA and DEPD exhibited strong activities in the presence of $Cu^{2+}$ or $Fe^{3+}$ ion, compared with VP-16 and PD. SA, DEPD, and VP-16 have two sites of binding, that is, free OH groups both at the 4′-position of the E-ring and in the polycyclic skeleton in their molecules. In the case of PD, however, the OH group at the 4′-position of the E-ring is masked by a methyl group. It is well known that the presence of the OH group at the 4′-position of the E-ring is essential for the exhibition of the cytotoxicity of PD-related compounds (43). For example, 4′-demethyl congeners of PD are more active than the other derivatives, in terms of antimicrotubule activity and microsomal protein binding (43). VP-16, but not PD, inhibits daunomycin-promoted microsomal lipid peroxidation, suggesting that the presence of the 4′-OH group is also necessary for the antioxidant effect of VP-16 (11, 17). Among the compounds possessing a 4′-OH group in their molecules, the metal complexes of VP-16 showed relatively low DNA-cleaving properties, compared with those of other compounds. It seems that the metal ion may bind to the sugar moiety in VP-16, thus decreasing the DNA-cleaving activity.

The OH group of the polycyclic skeleton in the PD-related compounds may be involved in intercalation with DNA molecules. The phenolic group of the E-ring may participate in complex formation with metal ions in cells. The metal ion bound with PD-related compounds would be reduced, and the resulting reduced metal ion or metal complex would then react with molecular dioxygen, thus producing an active oxygen species such as ·OH. The active oxygen species generated in this way would then attack the DNA strand. A proposed scheme for DNA cleavage by the metal complexes of PD-related compounds is illustrated in Fig. 9. In the PD-metal ion systems, the DNA breakage was found to be much less significant, but it was greatly promoted by UV irradiation. This suggests that the formation of demethyl derivatives may be involved. It has been reported that a catechol derivative, that is, the product of demethylation of the 3′-methoxy group of the E-ring, exhibits stronger DNA-cleaving activity than the parent compound (32). Recently, dihydroxy-etoposide, which is a metabolite of the clinically active VP-16, has been found to induce DNA cleavage in the presence of $Cu^{2+}$ ion, indicating that hydroxy radicals were responsible for the reaction (44). From a chemical viewpoint and based on our new findings and those reported for dihydroxy-etoposide (44) mentioned above, metal ion-induced DNA cleavage appears to be another powerful and innovative mechanism for understanding the antitumor activities of PD and its derivatives. It is also interesting to note that the hydroxy derivative of VP-16, which is a metabolite of VP-16, is cytotoxic to human breast tumor cells (32). We are now investigating the selectivity of DNA-cleaving action of these metal complexes, to find whether they recognize a singular DNA base.

**References**


1. Jardine, I. Podophyllotoxins, in *Anticancer Agents Based on Natural Product Models* (J. M. Cassady and J. D. Douros, eds.). Academic Press, New York, 319–351 (1980).
2. Issell, B. F., F. M. Muggia, and S. K. Carter. *Etoposide [VP-16]: Current Status and New Developments*. Academic Press, Orlando, FL, 1–355 (1984).
3. Stahelin, H., and A. von Wartburg. From podophyllotoxin glucoside to etoposide. *Prog. Drug Res.* **33**:169–266 (1989).
4. Levy, R. K., I. H. Hall, and K. H. Lee. Antitumor agents. LXII. Synthesis and biological evaluation of podophyllotoxin esters and related derivatives. *J. Pharm Sci.* **72**:1158–1161 (1983).
5. Beard, A. R., M. G. B. Drew, P. Hilgard, B. D. Hudson, J. Mann, S. Neidle, and L. F. T. Wong. Podophyllotoxin analogues: synthesis and computer modelling investigation of structure-activity relationships. *Anti-Cancer Drug Design* **2**:247–252 (1987).
6. Wilson, L., and M. Friedkin. The biochemical events of mitosis. II. The *in vivo* and *in vitro* binding of colchicine in grasshopper embryos and its possible relation to inhibition of mitosis. *Biochemistry* **6**:3126–3135 (1967).
7. Peter, J. O., L. J. Brian, A. Teresa, M. Silvia, and E. W. Robert. Drug therapy of etoposide (VP-16–213): current status of an active anticancer drug. *N. Engl. J. Med.* **312**:692–700 (1985).
8. Rozencweig, M., D. D. Hoff, J. E. Henney, and F. M. Muggia. VM 26 and VP 16–213: a comparative analysis. *Cancer (Phila.)* **40**:334–342 (1977).
9. Sinha, B. K., M. A. Trush, and B. Kalyanaraman. Free radical metabolism of VP-16 and inhibition of anthracycline-induced lipid peroxidation. *Biochem. Pharmacol.* **32**:3495–3498 (1983).
10. Sinha, B. K., M. A. Trush, and B. Kalyanaraman. Microsomal interactions and inhibition of lipid peroxidation by etoposide (VP-16,213): implications for mode of action. *Biochem. Pharmacol.* **34**:2036–2040 (1985).
11. Loike, J. D., and S. B. Horwitz. Effect of VP-16–213 on the intracellular degradation of DNA in HeLa cells. *Biochemistry* **15**:5443–5448 (1976).
12. Wozniak, A. J., and W. E. Ross. DNA damage as a basis for 4′-demethyl epipodophyllotoxin-9-(4,6-*O*-ethylidene-β-D-glucopyranoside) (etoposide) cytotoxicity. *Cancer Res.* **43**:120–124 (1983).
13. Sinha, B. K., and C. E. Myers. Irreversible binding of etoposide (VP-16–213) to deoxyribonucleic acid and proteins. *Biochem. Pharmacol.* **33**:3725–3728 (1984).
14. Haim, N., J. Roman, and B. K. Sinha. Peroxidative free radical formation and *O*-demethylation of etoposide (VP-16) and teniposide (VM-26). *Biochem. Biophys. Res. Commun.* **135**:215–220 (1986).
15. Haim, N., J. Nemec, J. Roman, and B. K. Sinha. *In vitro* metabolism of etoposide (VP-16–213) by liver microsomes and irreversible binding of reactive intermediates to microsomal protein. *Biochem. Pharmacol.* **36**:527–536 (1987).
16. Maanen, J. M. S., E. Akker, J. D. Vries, T. R. Bakkenist, J. Lankelma, J. Retel, and H. M. Pinedo. Structure-bioactivation relationship of a series of podophyllotoxin derivatives. *Eur. J. Cancer Clin. Oncol.* **24**:1415–1419 (1988).
17. Haim, H., J. Nemec, J. Roman, and B. K. Sinha. Peroxidase-catalyzed metabolism of etoposide (VP-16–213) and covalent binding of reactive intermediates to cellular macromolecules. *Cancer Res.* **47**:5835–5840 (1987).
18. Maanen, J. M. S., J. D. Vries, D. Pappie, E. Akker, M. V. Lafleur, J. Retel, J. Greef, and H. M. Pinedo. Cytochrome P-450-mediated *O*-demethylation: a route in the metabolic activation of etoposide (VP-16–213). *Cancer Res.* **47**:4658–4662 (1987).
19. Maanen, J. M. S., C. D. Ruiter, J. D. Vries, P. R. Kootstra, F. Gobas, and H. M. Pinedo. The role of metabolic activation by cytochrome P-450 in covalent binding of VP-16–213 to rat liver and HeLa cell microsomal proteins. *Eur. J. Cancer Clin. Oncol.* **21**:1099–1106 (1985).
20. Kalyanaraman, B., J. Nemec, and B. K. Sinha. Characterization of free

radicals produced during oxidation of etoposide (VP-16) and its catechol and quinone derivatives: an ESR study. *Biochemistry* **28**:4839–4846 (1989).
21. Ross, W., T. Rowe, B. Glisson, J. Yalowich, and L. Liu. Role of topoisomerase II in mediating epipodophyllotoxin-induced DNA cleavage. *Cancer Res.* **44**:5857–5860 (1984).
22. Tewey, K. M., G. L. Chen, E. M. Nelson, and L. F. Liu. Intercalative antitumor drugs interfere with the breakage-reunion reaction of mammalian DNA topoisomerase II. *J. Biol. Chem.* **259**:9182–9187 (1984).
23. Nelson, E. M., K. M. Tewey, and L. F. Liu. Mechanism of antitumor drug action: poisoning of mammalian DNA topoisomerase II on DNA by 4′-(9-acridinylamino)-methanesulfone-*m*-anisidide. *Proc. Natl. Acad. Sci. USA* **81**:1361–1365 (1984).
24. Sakurai, H., M. Shibuya, C. Shimizu, S. Akimoto, M. Maeda, and K. Kawasaki. DNA strand scission by hemin-thiolate complexes as models of cytochrome P-450. *Biochem. Biophys. Res. Commun.* **136**:645–650 (1986).
25. Shibuya, M., H. Sakurai, T. Maeda, E. Nishiwaki, and M. Saito. Synthesis of the degradation product of auromomycin chromophore and DNA-cleaving activities of its derivatives. *Tetrahedron Lett.* **27**:1351–1354 (1986).
26. Nishiwaki, E., H. Lee, T. Matsumoto, K. Toyooka, H. Sakurai, and M. Shibuya. Synthesis of nitrated oligo-*N*-methylpyrrole carboxamide derivatives and their photochemical DNA-cleaving activities. *Tetrahedron Lett.* **31**:1299–1302 (1990).
27. Thurston, L., H. Irie, S. Tani, F. S. Han, Z. C. Liu, Y. C. Cheng, and K. H. Lee. Antitumor agents. 78. Inhibition of human DNA topoisomerase II by podophyllotoxin and α-peltatin analogues. *J. Med. Chem.* **29**:1547–1550 (1986).
28. Thurston, L., Y. Imakura, M. Haruna, D. H. Li, Z. C. Liu, Y. C. Cheng, and K. H. Lee. Antitumor agents. 100. Inhibition of human DNA topoisomerase II by cytotoxic ether and ester derivatives of podophyllotoxin and α-peltatin. *J. Med. Chem.* **32**:604–608 (1989).
29. Kivelson, D., and R. Neiman. ESR studies on the bonding in copper complexes. *J. Chem. Phys.* **35**:149–155 (1961).
30. Bryce, G. F. Electron paramagnetic resonance study of cupric-peptide complexes. *J. Phys. Chem.* **70**:3549–3557 (1966).
31. Gersmann, H. R., and J. D. Swalen. Electron paramagnetic resonance spectra of copper complex. *J. Chem. Phys.* **36**:3221–3233 (1962).
32. Maki, A. H., and B. R. McGarvey. Electron spin resonance in transition metal chelates. I. Copper(II) bis-acetylacetonate. *J. Chem. Phys.* **29**:31–34 (1958).
33. Maki, A. H., and B. R. McGarvey. Electron spin resonance in transition metal chelates. II. Copper(II) bis-salicylaldehyde-imine. *J. Chem. Phys.* **29**:35–38 (1958).
34. Bachmayer, H., L. H. Piette, K. T. Yasunobu, and H. R. Whiteley. The binding sites of iron in rubredoxin from *Micrococcus aerogenes*. *Proc. Natl. Acad. Sci. USA* **57**:122–127 (1967).
35. Kalyanaraman, B., R. C. Sealy, and B. K. Sinha. An electron spin resonance study of the reduction of peroxides by anthracycline semiquinones. *Biochim. Biophys. Acta* **799**:270–275 (1984).
36. Doroshow, J. H., and K. J. Davies. Redox cycling of anthracyclines by cardiac mitochondria. *J. Biol. Chem.* **261**:3068–3074 (1986).
37. Sinha, B. K., H. M. Eliot, and B. Kalyanaraman. Iron-dependent hydroxyl radical formation and DNA damage from a novel metabolite of the clinically active antitumor drug VP-16. *FEBS Lett.* **227**:240–244 (1988).
38. Schneider, J. E., M. M. Browning, and R. A. Floyd. Ascorbate/iron mediation of hydroxyl free radical damage to PBR 322 plasmid DNA. *Free Radical Biol. Med.* **5**:287–295 (1988).
39. Chikira, M., W. E. Antholine, and D. H. Petering. Orientation of dioxygen bound to cobalt(II) bleomycin-DNA fibers. *J. Biol. Chem.* **264**:21478–21480 (1989).
40. Solomon, L. R., R. D. Beerelli, and P. L. Moseley. Bleomycin-iron can degrade DNA in the presence of excess ethylenediaminetetra-acetic acid *in vitro*. *Biochemistry* **28**:9932–9937 (1989).
41. Sugiura, Y. Bleomycin-iron complexes: electron spin resonance study, ligand effect, and implication for action mechanism. *J. Am. Chem. Soc.* **102**:5208–5215 (1980).
42. Bailly, C., A. Kenani, N. Helbecque, J. Bernier, R. Houssin, and J. Henichart. DNA-binding and DNA-cleaving properties of a synthetic model AGAGLU related to the antitumor drugs AMSA and bleomycin. *Biochem. Biophys. Res. Commun.* **152**:695–702 (1988).
43. Long, B. H., S. T. Musial, and M. G. Brattain. Comparison of cytotoxicity and DNA breakage activity of congeners of podophyllotoxin including VP16–213 and VM 26: a quantitative structure-activity relationship. *Biochemistry* **23**:1183–1188 (1984).
44. Sinha, B. R., W. M. Autholine, and B. Kalyanuraman. Copper ion-dependent oxy-radical mediated DNA damage from dihydroxy derivative of etoposide. *Biochim. Biophys. Acta* **1096**:81–83 (1990).

**Send reprint requests to:** Dr. H. Sakurai, Kyoto Pharmaceutical University, Nakauchi-cho 5, Misasagi, Yamashina-ku, Kyoto 607, Japan, or Dr. K. H. Lee, Natural Products Laboratory, School of Pharmacy, University of North Carolina, Chapel Hill, NC 27599–7360.