Breaks in Double-Strand DNA by Cu(II) Complexes of Etoposide (VP-16) and Its Derivatives, as Evaluated by S1 Nuclease Treatment

Riichi TAWA,*[,a] Masashi TAKAMI,[a] Yasuhiro IMAKURA,[b] Kuo-Hsiung LEE,[c] and Hiromu SAKURAI*[,a]

Department of Analytical and Bioinorganic Chemistry, Kyoto Pharmaceutical University,[a] Misasagi, Yamashina-ku, Kyoto 607, Japan, Faculty of Science, Naruto University of Education,[b] Tokushima 772, Japan and Division of Medicinal Chemistry and Natural Products, School of Pharmacy, University of North Carolina,[c] Chapel Hill, NC 27599, U.S.A.
Received May 2, 1997; accepted June 16, 1997

Single-strand breaks (ssb) in double-strand (ds) DNA produced by hydroxyl radicals (·OH) generated by Cu(II) complexes of podophyllotoxin (PD)-related compounds were evaluated using S1 nuclease digestion. Cu(II) complexes of VP-16 (etoposide, 4′-demethylepipodophyllotoxin-9-(4,6-*O*-ethylidene-β-D-glucopyranoside)), 4′-demethylepi-PD (DEPD), and syringic acid (SA) exhibited both ssb and ds breaks (dsb) in ColE1-*Hae*II and pBR322-*Bgl*I DNA fragments, in which the number of ssb was found to be more than three times and four times greater than that of dsb, respectively. Cytosine (C)-methylation of cytosine-guanine doublet (CpG) in pBR322-*Bgl*I DNA inhibited both ssb and dsb within DNA segments by ·OH generated by the Cu(II) complexes.

Key words VP-16 (etoposide); copper(II) ion; DNA strand breaks; hydroxyl radical (·OH); S1 nuclease; 5-methylcytosine

Podophyllotoxin (PD) and many of its closely related lignans have been recognized as potent inhibitors of cell mitosis.[1] The action mechanism of VP-16 (etoposide, 4′-demethylepipodophyllotoxin-9-(4,6-*O*-ethylidene-β-D-glucopyranoside)) has been shown to be different from that of PD, although their structures are closely related[2] (Fig. 1). In the mechanism of antitumor activity of VP-16, an interaction between the drug and topoisomerase II has been suggested to result in a covalent enzyme–DNA complex, which in turn produces double-strand breaks (dsb) in the DNA.[3] The metabolizing enzymes such as cytochrome P450, peroxidases and tyrosinase have been shown to transform VP-16 into forms more cytotoxic against tumor cells.[4–6] For instance, the *ortho*- and semi-quinone free radicals, as the intermediates, bind more strongly than VP-16 to DNA, inducing DNA single-strand break (ssb) and dsb.[7] Sinha *et al.*[8] 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. We have found that ·OH can be generated during a redox reaction-dependent complex formation between Cu(II) ion and VP-16, 4′-demethylepi-PD (DEPD), and syringic acid (SA) in the presence of molecular dioxygen (O_2), and that ·OH participates in the strand breaks of ColE1 plasmid DNA[9] and M13mp18 single-strand (ss)-DNA.[10] The damage on the M13mp18 ss-DNA (7249 base) and the ColE1/*Taq* I-*Bst*XI double-strand (ds)-fragment (2248 bp) produced by the Cu(II) complexes were also assessed by a modified polymerase chain termination assay[10] and the Maxam and Gilbert method,[11] respectively, in which they cleaved the site specifically at pH 7.8 at both guanine (G) and cytosine (C) positions in the GC rich regions of the ss-DNA or G positions of the ds-DNA fragments, respectively. These results suggest that the primary sequence of DNA affects both the binding and the interaction of the Cu(II) complexes.

S1 nuclease from *Aspergillus oryzae*, which is an endonuclease for DNA, exhibits high selectivity for ss-DNA and hydrolyzes ss regions in duplex DNA.[12,13] This enzyme also detects locally altered DNA structures induced by physical and chemical procedures.[14] For example, DNA dsb have been observed following incubation of γ-irradiated DNA with S1 nuclease.[15] These dsb are attributed to cleavage opposite ssb and to localized DNA denaturation caused by radiation-induced base damage or possibly base losses. Therefore, this enzyme could be used to evaluate a strand break opposite a nick in the chemically induced disruption of DNA.

DNA possesses different amounts of 5-methylcytosine (5mC) as a covalently modified base of DNA.[16–18] It has been reported that tumor cellular DNA is undermethylated compared with normal cellular DNA but the extent varies with the type of tumor.[19] 5mC facilitates the B to Z transition *in vitro* in synthetic linear DNA polymers,[20]

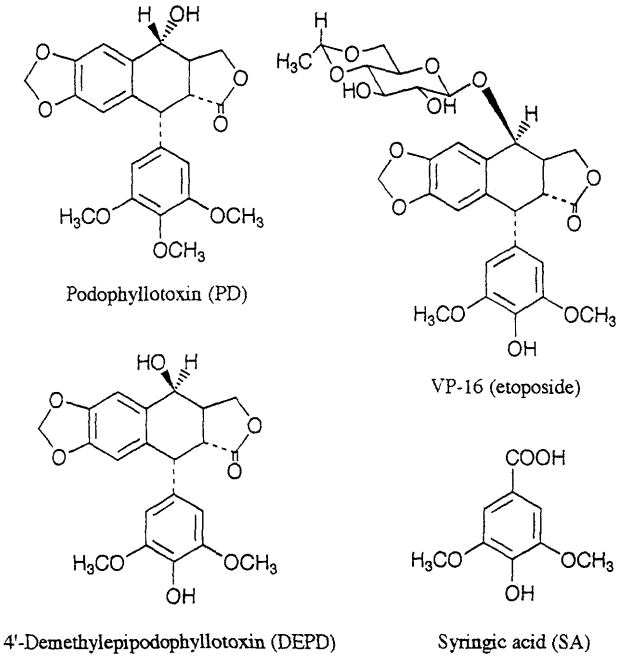

Fig. 1. Structures of PD, VP-16, DEPD and SA

* To whom correspondence should be addressed.

and thus is effective in stabilizing Z-DNA conformation.[21] Most 5mC occurs at C-G doublet (CpG) of DNA.[22] We earlier investigated the neighboring effects of 5mC to the G-specific dsb in DNA, especially on methylated CpG by ·OH, and found that C-methylation in CpG pairs is an important factor for inhibition of G-dependent dsb by the Cu(II) complexes.[11]

The purpose of this study was to estimate the ssb in the unmethylated and methylated plasmid DNA fragments produced by ·OH attack which was generated by VP-16, DEPD, and SA in the presence of Cu(II) ion and O_2, using S1 nuclease digestion. This is the first report of the application of S1 nuclease to determine both ssb and dsb in unmethylated and methylated DNA by ·OH generated from the Cu(II)–drug complex.

MATERIALS AND METHODS

Materials DNA fragments were prepared from plasmid pBR322 (Nippon Gene, Tokyo) and ColE1 (Nippon Gene, Tokyo) digested with *Bgl*I (Nippon Gene, Tokyo) and *Hae*II (Toyobo, Osaka), respectively. pBR322-*Hin*dIII (Takara, Kyoto) fragment (4363 bp) was prepared as specified by the manufacturers. VP-16 and SA were obtained as commercial products (Sigma, St. Louis, MO and Tokyo Kasei, Tokyo, respectively). DEPD was prepared as described previously.[9,11] VP-16, DEPD, and SA were dissolved in a mixture of acetonitrile and TA (20 mM Tris–acetate, pH 7.8) at 4:1. All other chemicals and the purified water used were obtained as described.[11]

***In Vitro* Methylation of Plasmid DNA Fragments** C methylation of CpG pairs in pBR322-*Bgl*I fragments (234, 1810, and 2319 bp) was carried out as described.[11] After phenol extraction and ethanol precipitation,[18] CpG methylation was analyzed by digestions with *Msp*I (CCGG, Toyobo, Osaka) and *Hpa*II (isoschizomer of *Msp*I, CCGG, Toyobo, Osaka) and the following 0.8% agarose gel electrophoresis in 1x TAE (40 mM Tris–acetate, 1 mM EDTA, pH 8.0). The populations were almost completely (>99%) resistant to cleavage by *Hpa*II (data not shown).

DNA Cleavage Reaction The unmethylated ColE1-*Hae*II fragments (0.5 μg) or the methylated and unmethylated pBR322-*Bgl*I fragment (0.5 μg) were incubated in a reaction mixture containing Cu(II) ion (0.5 mM $CuCl_2$) plus VP-16, DEPD, or SA in TA (pH 7.8) at 37 °C. Ligand and Cu(II) ion concentrations are given in Tables 1 and 2. Reactions were started by addition of the ligand and stopped after 3 h for the VP-16- and DEPD-containing systems or after 1 h for the SA-containing system by the addition of 2 μl of a terminating agent containing 40% sucrose and 0.25% bromophenol blue. The induced breaks for the ColE1-*Hae*II fragments (1317 bp) and the pBR322-*Bgl*I fragment (2391 bp) were analyzed by 0.8% agarose-gel electrophoresis as described above. For evaluation of dsb induced by Cu(II) complexes, the gels were stained with ethidium bromide (0.5 μg/ml), photographed with a short wavelength UV light (303 nm), and the fluorescence of each gel was determined by densitometric measurements at 285 nm excitation wavelength (Shimadzu CS-9000, Kyoto).

S1 Nuclease Digestion After addition of pBR322-*Hin*dIII fragment (0.213 μg) as the quantitative standard into the reaction samples and its precipitation with ethanol, the samples were digested with 5 units of S1 nuclease (Takara, Kyoto) per 0.5 μg DNA and were incubated for 30 min at 37 °C. The reactions were terminated by cooling the mixtures on ice, followed by the addition of 2 μl of the terminating agent as described above. After the samples were separated by 0.8% agarose gel electrophoresis, ssb and dsb induced in DNA fragments by the Cu(II) complexes were evaluated.

Statistics Statistical analysis was performed by Student's *t* test for comparison between ssb and dsb. A probability of 0.05 or less was considered to be significant.

RESULTS AND DISCUSSION

VP-16 has been shown to cause both ssb and dsb in DNA of tumor cells or their isolated nuclei.[23] During DNA replication in the S-phase of the cell cycle, extensive strand separation takes place, and DNA is present in the ss form at many replication forks in tumor cells.[24] Therefore, if these ss sites are accessible to ·OH attack, considerable inhibition of DNA replication can be expected. DNA dsb is also of interest, because they are generally considered to be more lethal lesions to tumor cells. The present work focused on the evaluation of ssb in purified plasmid DNA fragments produced by ·OH generated from PD-related compounds in the presence of Cu(II) ion and O_2, as well as on whether C-methylation in CpG pairs is also an important factor in the inhibition of DNA ssb by these complexes.

S1 nuclease has been widely used to probe structural transitions in DNA.[12] The ability of S1 nuclease to cleave opposite a nick on DNA is well known and it cleaves ss-DNA at 3′-phosphate phosphodiester bonds but shows no base-specific recognition pattern.[13] Therefore, treatment of ds-DNA with S1 nuclease allowed to map the sites on DNA ssb produced locally by ·OH generated by Cu(II) complexes of VP-16, DEPD, or SA (Fig. 2) and to evaluate each of ssb and dsb in the DNA. The number of ssb and dsb in ColE1-*Hae*II (1317 bp) and pBR322-*Bgl*I DNA fragment (2391 bp) produced by Cu(II) complexes is summarized in Tables 1 and 2, respectively. Loike and Horwitz observed that VP-16 did not produce any DNA damage in a purified type 2 adenovirus DNA.[24] A 0.5 mM Cu(II) ion alone also did not induce any strand breakages in ColE1-*Hae*II and pBR322-*Bgl*I DNA fragments under the conditions used.[10] However, the present data indicate that the Cu(II) complexes of VP-16-related compounds involving VP-16 exhibit both ssb and dsb on the plasmid DNA fragments, although VP-16 complex did not show highly significant differences ($p<0.1$ for 5 mM VP-16 plus 0.5 mM Cu(II)). The number of ssb produced in ColE1-*Hae*II fragment by Cu(II) complexes of DEPD was three times higher than dsb, although no difference was found in DNA cleavage by the Cu(II)–VP-16 complex. On the other hand, the number of ssb produced in pBR322-*Bgl*I fragment by Cu(II) complexes was almost four times that of dsb, as shown in Table 2. Our data

indicate that nicks within ColE1-*Hae*II and pBR322-*Bgl*I fragments destabilize the DNA sufficiently to allow digestion by S1 nuclease. Although S1 nuclease is specific for ss-DNA, DNA with locally altered structures (minor distortions, transient unwinding, *etc.*) is also recognized by this enzyme leading to DNA strand break formation.[13] For example, this nuclease recognizes junctions between right-handed B- and left-handed Z-DNA, although the location of the cleavage sites differs between junctions.[12] It is also known that *in vitro* C-methylation also facilitates the B to Z transition in synthetic linear DNA polymers,[25,26] and in certain sequences in supercoiled recombinant plasmids.[27] It has further been reported that the relative efficiency of bleomycin cleavage, which is induced by radical-based reactions in the presence of metal ions *in vitro*,[28] is often reduced at sites that have been methylated by restriction methylases.[29] We found that CpG methylation of the DNA fragments by M.*Sss*I modification significantly decreased in DNA dsb.[11] Thus, we also examined the effect of 5mC on ssb in the pBR322-*Bgl*I fragments by the Cu(II) complexes (Fig. 2 and Table 2). Considering that S1 nuclease induces dsb only in the DNA sequence containing abasic site and nick, the number of ssb in Table 2 showed that 5mC as a neighboring base at G also decreased in ssb for DNA produced by the Cu(II) complexes. When Student's *t* test was applied, significant differences between ssb and dsb, which were produced by the Cu(II) complexes of VP-16 and DEPD for both unmethylated and methylated pBR322-*Bgl*I DNA fragments, were observed as $p<0.01$ and $p<0.0001$, respectively. The decrease in ssb and especially in dsb on the hypermethylated DNA fragments may be due to local conformational changes of DNA resulting from C-modification,[20] *i.e.*, the differences of ssb and dsb between the unmethylated and methylated pBR322-*Bgl*I fragment may reflect the existence of smaller Z-DNA segments within the methylated fragments as well as conformational heterogeneity. The precise nature of DNA structure, however, recognized by S1 nuclease remains to be elucidated. C-methylation of the G·C base pair sites has also been reported to reduce the affinity of Cu(II) ion for duplex DNA, as evidenced by a lessening of the destabilizing effect of Cu(II) on the melting of DNA.[30] Therefore, the 5mC residue was concluded to be an important factor in inhibiting G-dependent ssb within a DNA segment by ·OH generated by Cu(II) complexes of PD and its related compounds.

In conclusion, Cu(II) complexes of VP-16, DEPD, and SA, that in turn generate ·OH, cleaved both ss- and ds-DNA, especially with three-fold higher ssb in number than that of dsb in ColE1-*Hae*II DNA fragment by Cu(II) complex of DEPD, and with ssb of almost four times dsb in pBR322-*Bgl*I DNA fragment by all Cu(II) complexes,

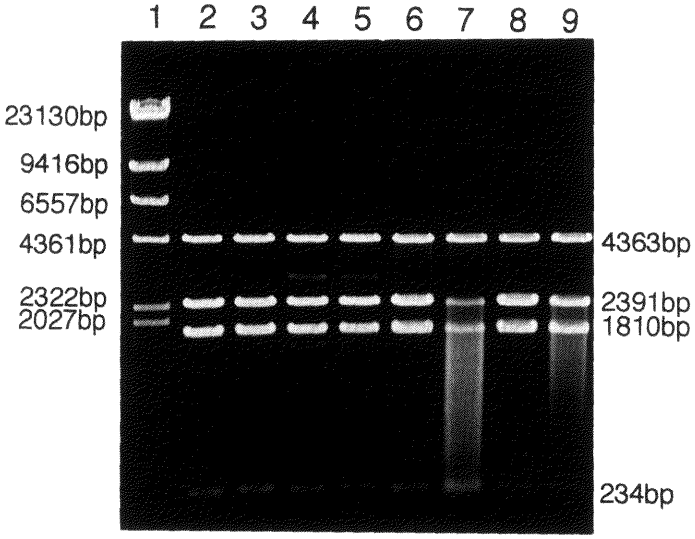

Fig. 2. Effects of CpG Methylation on ssb of pBR322-*Bgl*I Fragment by VP-16 in the Presence of Cu(II) Ion

0.5 μg of pBR322-*Bgl*I fragment was incubated at 37 °C for 3 h. Lanes 6, 7, 8, and 9 were incubated with 5 units of S1 nuclease at 37 °C for 30 min after the cleavage reaction. Lane 1, λ-*Hin*dIII 0.5 μg; 2 and 6, pBR322-*Bgl*I fragment (2391 bp); 3 and 7, the DNA fragments reacted with 10 mM VP-16 and 0.5 mM Cu(II) ion; 4 and 8, the methylated pBR322-*Bgl*I fragments (2391, 1810 and 234 bp); 5 and 9, the methylated fragment reacted with 10 mM VP-16 and 0.5 mM Cu(II) ion, respectively. The 4363 bp DNA in lanes 2—9 is pBR322-*Hin*dIII fragment and was used as a marker for the concentration.

Table 1. Estimation of the Percentage of ssb and dsb in ColE1/*Hae*II Fragments Produced by VP-16, DEPD, and SA in the Presence of Cu(II) Ion and O_2[a]

	ssb (%) (*n*)[b]	dsb (%) (*n*)
VP-16 10 mM + Cu(II) 0.5 mM	30.8 ± 15.5 (4)	29.3 ± 4.3 (4)
VP-16 5 mM + Cu(II) 0.5 mM	29.3 ± 25.9 (4)	1.8 ± 3.5 (4)
DEPD 10 mM + Cu(II) 0.5 mM	83.8 ± 17.6** (4)	15.0 ± 18.5 (4)
DEPD 5 mM + Cu(II) 0.5 mM	86.7 ± 15.9♯ (3)	3.3 ± 5.8 (3)
SA 5 mM + Cu(II) 0.5 mM	29.7 ± 13.9* (3)	70.3 ± 13.9 (3)
SA 0.5 mM + Cu(II) 0.5 mM	39.7 ± 33.6 (3)	11.3 ± 12.0 (3)

a) Reactions were carried out at 37 °C for 1 h (SA/Cu), 3 h (VP-16/Cu and DEPD/Cu), respectively. Each value is the mean ± SD of *n* independent experiments which was calculated by monitoring of 1317 bp of ColE1/*Hae*II fragments. *b*) After the reaction, 5 units of S1 nuclease/0.5 μg DNA was added to evaluate only ssb in DNA. Asterisks, significant change from the corresponding dsb (*, $p<0.05$; **, $p<0.005$; ♯, $p<0.001$).

Table 2. Estimation of the Percentage of ssb and dsb in Unmethylated and Methylated pBR322-*Bgl*I Fragment with/without S1 Nuclease Treatment[a]

	Unmethylated fragmen (%)[b]		Methylated fragment (%)[b]	
	ssb (*n*)[c]	dsb (*n*)	ssb (*n*)[c]	dsb (*n*)
VP-16 10 mM + Cu(II) 0.5 mM	66.1 ± 3.4** (4)	17.6 ± 4.9 (4)	38.1 ± 3.0** (3)	0.6 ± 0.98 (3)
DEPD 10 mM + Cu(II) 0.5 mM	74.8 ± 1.9** (3)	22.3 ± 2.2 (3)	49.9 ± 8.2** (4)	0 ± 3.7 (4)
SA 0.5 mM + Cu(II) 0.5 mM	064.8 ± 12.1** (5)	18.2 ± 9.4 (5)	29.7 ± 7.5* (3)	2.5 ± 4.4 (3)

a) Reactions were carried out at 37 °C and pH 7.8 for 1 h (SA/Cu) and 3 h (VP-16/Cu and DEPD/Cu), respectively. *b*) Each value is the mean ± S.D. of *n* independent experiments which was calculated by monitoring 2391 bp of pBR322/*Bgl*I fragments. *c*) After the reaction, 5 units of S1 nuclease/0.5 μg DNA was added to evaluate only ssb in DNA. Asterisks, significant change from the corresponding dsb (*, $p<0.01$; **, $p<0.0001$).

respectively. In addition, C-methylation at CpG pairs in DNA was found to be important for diminishing the ·OH-dependent DNA ssb. The present and the previous results should be very useful for developing new antitumor compounds in relation to the structure and conformation of DNA of tumor cells.

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