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EFFECTS OF CpG METHYLATION TO DOUBLE STRANDED DNA BREAKS BY Cu(II)-PODOPHYLLOTOXIN DERIVATIVE COMPLEXES

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Abstract: Site-specific double stranded DNA breaks by •OH radicals generated from podophyllotoxin related compounds in the presence of Cu(II) ion were investigated and the neighboring effects of 5-methylcytosine on double stranded DNA cleavage, especially on methylated CpG sites, were evaluated.

VP16 (etoposide) and VM26 (teniposide) are both semi-synthetic derivatives of podophyllotoxin (PD), which is a natural product isolated from certain plants of the genus podophyllum[1], and clinically used as cancer chemotherapeutic agents. Their action mechanisms are suggested to be different from that of PD, but being yet unclear[2]. Loike & Horwitz[3], Wozmiak & Ross[4], and Long et al.[5] reported that the cytotoxicity of these compounds is due to DNA damage in L1210 and human lung adenocarcinoma cells. While, the fact that VP16 and VM26 give a direct radical-dependent DNA damage to the cells has attracted considerable interest as one of the mechanisms for their antitumor activities[6].

As suggested in the previous paper[7], Cu(II) ion bound with 4'-demethylepipodophyllotoxin-9-(4,6-O-ethylidene-β-D-glucopyranoside) (VP16), 4'-demethylepipodophyllotoxin (DEPD) and syringic acid (SA) as PD related compounds (Fig. 1) is reduced, and the resulting Cu(I) ion or its complexes would then react with molecular dioxygen, thus producing hydroxyl radicals (•OH) which induce the nicking of plasmid DNA. We also demonstrated that the •OH participates in the strand break formation produced by Cu(II)-complexes from the

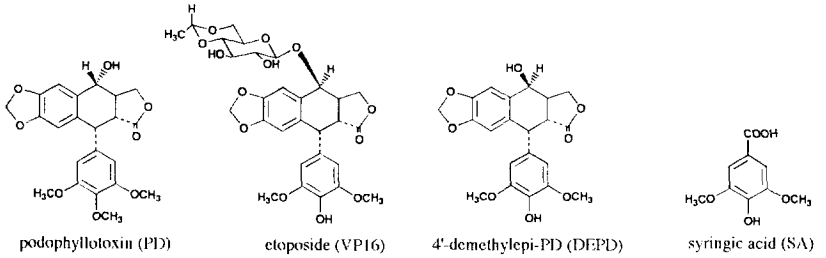

Fig. 1

results on •OH scavengers to protect DNA from damage . In addition, our data suggested that a free hydroxyl group at the 4'-position of VP16 and DEPD is essential for DNA breakage. The damage produced by Cu(II)-complexes was also assessed by a polymerase chain termination assay[8]. Cu(II)-complexes of DEPD or SA cleaved M13mp18ssDNA site-specifically at both guanine and cytosine positions in the GC rich regions, respectively, at pH 7.8. These results suggest that the primary sequence of M13mp18DNA affects the binding and interaction of Cu(II) complexes with SA or DEPD.

5-Methylcytosine (5mC) is a covalent modification of DNA and mostly occurs at CpG sites[9-11]. The 5mC is proposed to act as an important switch for gene regulation[12-15]. In addition, cytosine 5-methylation facilitates the B to Z transition *in vitro* in synthetic linear DNA polymers[16] and thus is effective in stabilizing Z-DNA conformation by reducing the amount of negative supercoiling to stabilize the Z conformation[17]. Furthermore, it has been proved that cytosine 5-methylation dramatically lowers the B-Z transition energy of the d(CG) dinucleotide[18], and bleomycin-mediated strand scission decreases substantially in proximity to the methylated cytosine moieties in DNA[19]. On the basis of these observations, we have extended our study to know the site-specific single stranded cleavage in double stranded DNA by DEPD, SA and VP16 in the presence of Cu(II) ion, characterizing the effect of 5mC as a neiboring base on DNA cleavage at guanine site. Although the nearest neighbor effects on carcinogen binding to guanine have already been determined for a number of unique sequences and direct acting carcinogens[20-22], little work has been done on the effect of 5mC to the DNA cleavage of the double stranded DNA sequences. The present work has been focused on whether cytosine methylation in CpG pairs is an important factor to inhibit DNA cleavage by active oxygen species generated from the antitumor agents.

DNA fragments were prepared from plasmid pBR322 (Nippon Gene) and ColE1 (Nippon Gene) digested with *Taq* I (NEB) or *Bgl* I (Nippon Gene). VP16 was purchased as a commercial product (Sigma). DEPD was prepared as described previously[7]. All chemicals and water used were obtained as described in the previous report[8]. Methylation of pBR322-*Bgl* I fragments (234, 1810 and 2319 bp) by M.*Sss* I (NEB) was carried out in 10 mM Tris-HCl, pH 7.9 containing 50 mM NaCl, 1 mM DTT and 10 mM of $MgCl_2$ containing 160 μM S-adenosylmethionine. After phenol extraction and ethanol precipitation, the methylation of cytosine in CpG pairs by *Sss* I methylase was analysed by the restriction enzyme digestions and 0.8% agarose gel electrophoresis in 1 x TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). The populations were completely (>99%) resistant to cleavage by *Hpa* II (CCGG, Toyobo), or *Acc* II (CGCG, Nippon Gene) restriction enzymes. The nonmethylated or methylated pBR322-*Bgl* I fragment (0.5 μg) was incubated in a reaction mixture containing various concentrations of Cu(II) ions plus SA, DEPD, PD or VP16 in 20 mM Tris-acetate buffer, pH 7.8, at 37 °C. Ligands and Cu(II) ion concentrations are given in the legends of the figures. Reactions were started by addition of the ligand and stopped after 3 hrs for VP16- and DEPD-containing systems or 1 hr for SA-containing system by addition of 2 μl of a terminating agent containing 40% sucrose and 0.25% bromophenol blue. The induced breaks for pBR322-*Bgl* I were analyzed on 0.8% agarose-gel electrophoresis with 1 x TAE as a running buffer. The gels were stained with ethidium bromide (0.5 μg/ml) and photographed with a short wavelength UV light (303 nm). The plasmid ColE1 was digested with *Taq* I and the resulting DNA fragments were fractionated by electrophoresis on 6% polyacrylamid gel. Aliquots of this digested DNA samples were

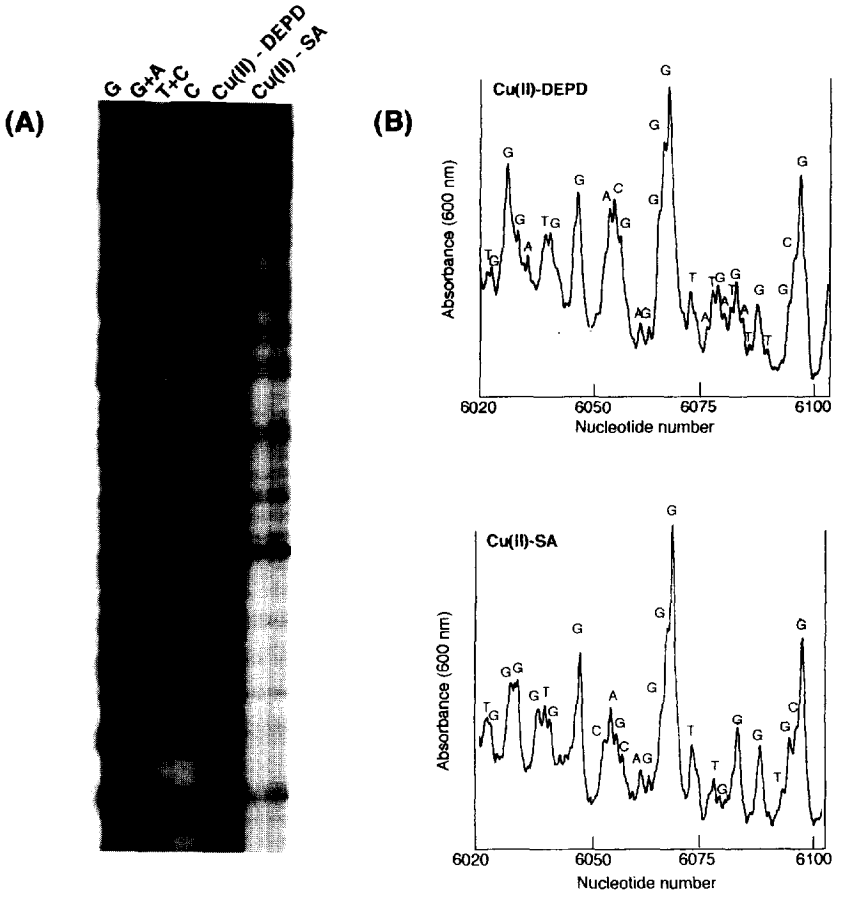

Fig. 2. Site specificity of double stranded DNA (plasmid ColE1) by Cu(II)-complexes with DEPD and SA. (A) Autoradiogram of sequencing gel of the 5'-end labeled *Taq* I-*Bst* XI fragment (2248 bp) of ColE1 DNA reacted with Cu(II)-complexes. DEPD-Cu(II): DEPD (10 mM) plus Cu(II) ion at 0.1 mM; SA-Cu(II): SA (5 mM) plus Cu(II) ion at 0.5 mM. Lanes G, G+A, T+C and C refer to Maxam and Gilbert sequencing reaction of the *Taq* I-*Bst* XI fragment of ColE1. (B) Densitometer scan of the autoradiogram.

loaded on a 0.8% agarose gel, and the remaining samples were used for the sequence determination by the method of Maxam and Gilbert[23]. The autoradiogram for a 6% gel electrophoresis was measured by densitometric measurements (Shimadzu CS-9000).

The autoradiogram determined by the sequencing analysis is shown in Fig. 2A, which was converted to a histogram (Fig. 2B) to identify the DNA cleavage sites. The most preferred site was formed on the guanine residue of the sequence, being consistent with our previous results that guanines and cytosines in the single stranded DNA are preferred sites by •OH radical attack[8]. DNA damage induced by the reactive Cu(II)- complexes is likely to occur in the immediate vicinity of the copper-binding sites[24]. Therefore, it readily accounts for the cutting pattern observed for DNA in the presence of Cu(II) ion and reducing species. Nakayama et al.[25] reported that the energy level of the highest occupied molecular orbital of guanine is the highest among the nucleic acid bases, and accordingly, guanine is oxidized most easily. Guanine residues are the most electronegative regions of the DNA molecule[26]. These models together with the data on the susceptibility of bases to oxidative damage are useful to explain the observed sequence dependence for Cu(II)-induced oxidative cleavage of double stranded DNA in solution. This fact is further supported by previous evidence showing an occurrence for binding of Cu(II) with guanine[27,28]. On the other hand, Yamamoto and Kawanishi[29] have shown that DNA damage induced by a Cu(II)/H_2O_2 system is sequence-dependent with bases, particularly thymines that are 5' to one or more adjacent guanine residues, being the most susceptible to damage. Thus, the specificity observed for the DNA cleavage reaction may depend on how the Cu(II) ion binds with bases in double stranded DNA and on the coordination geometry of the copper complexes as they relate to the positions of the atoms susceptible to oxidative damage.

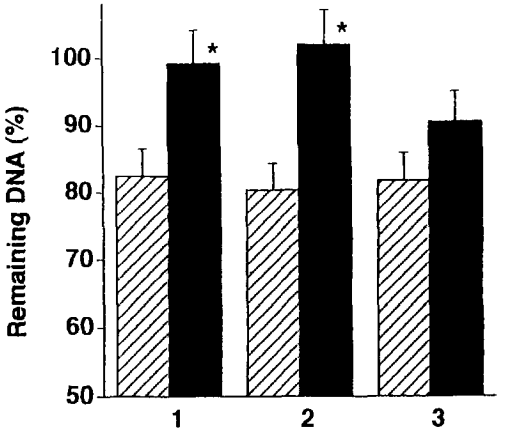

Fig. 3. Evaluation of the double strand breaks of nonmethylated and methylated in CpG doublets of plasmid pBR322-*Bgl* I fragment (2319 bp) by Cu(II)-complexes of VP16, DEPD and SA. 1: Cu(II) (0.5 mM)-VP16 (5 mM), 2: Cu(II) (0.5 mM)-DEPD (5 mM), 3: Cu(II) (0.5 mM)-SA (0.5 mM). Reactions were carried out for 3 (VP16), 3 (DEPD) and 1 hr (SA) at 37 °C and pH 7.8. Data represent the means of four independent experiments. Bars show the standard deviations of means. *: $P<0.01$ significantly different (Student's *t* test).

The results comparing with the effect of 5mC neighbors on DNA cleavage at guanine by the Cu(II)-complexes of VP16, DEPD or SA are shown in Fig. 3. Quantitative densitometric analysis indicated that 5mC at a 5' neighbors to guanine after *Sss* I modification significantly decreased double stranded DNA scission. Statistically significance of differences between nonmethylated and methylated DNA was evaluated by applying Student's two-tailed *t* test and p values of less than 0.01 were observed for VP16- and DEPD-complexes with Cu(II) ion. The susceptibility of DNA fragments to VP16-, DEPD- and SA-complexes were decreased by 16.8 ($p<0.01$), 19.6($p<0.01$) and 8.5%, respectively, with 5-mC at a 5' neighbor to guanine. The decreased strand scission may be due, in part, to local conformational changes of the DNA to Z-form (or other non-B-form structures) resulted by cytosine methylation[16]. In addition, modification of the G•C base pairs by methylating cytosine of DNA has been reported to reduce the affinity of DNA for Cu(II) ion as evidenced by a lessening of the destabilizing effect of Cu(II) on the melting of DNA[28]. Also, it generally accepted that Cu(II) ion binds to guanine sites in DNA has been obtained[30]. Ropars and Viovy[31] found that Cu(II) ion binds to the phosphate groups as well as to guanine through the C6OH and N7 groups in Cu(II)-DNA interactions by ESR study. Similarly, Tu and Friederich[32] have also confirmed that Cu(II) ion binds to the N7 and C6O groups of the guanine base of DNA by a conductometric and infrared studies. Therefore, these models suggest that Cu(II) complexes of PD related compounds appear to bind at the guanine residues in DNA as well as the property of Cu(II) ion.

In the present study we investigated the neighboring effects of 5mC in cleavage on double stranded DNA, which is methylated at CpG sites, by Cu(II) complexes. Cytosine methylation in CpG pairs was concluded to be an important factor for understanding the inhibition of guanine-dependent scission within a DNA segment. This evidence suggests that the recognition of specific methylation patterns could be a possible mechanism for selective chemotherapeutic action by DNA-damaging antitumor agents, such as VP16 or DEPD in the presence of Cu(II) ion. The results presented in this paper, together with those previously obtained by us[7,8], indicated a presence of an alternative mechanism for the action of VP16 based on its bioactivation to DNA damaging metabolites[33,34]. It has not been known how Cu(II) ion would become available for this free radical-dependent DNA damage *in vivo*. This damage is thought to be a result of oxygen radicals generated by the redox properties of the copper centers. Molecular dioxygen reacts with the Cu(I) formed, either still bound or in the proximity of DNA, which in turn generates •OH and Cu(II) in the presence of active oxygen species like H_2O_2, similarly to the Fenton-like reaction ($Cu(I) + H_2O_2 \rightarrow Cu(II) + \bullet OH + OH^-$). In addition, the present work suggests that cytosine methylation in CpG pairs alone is one factor to consider in the inhibition of double stranded breaks by •OH generated from Cu(II)-complexes of the VP16 and its related compounds. If the observed diminution of Cu(II)-complex-mediated DNA strand scission was simply due to an unfavorable steric interaction between the 5-methyl group of cytosine and some portion of the VP16 or its related compounds, the effect would be expected to be most pronounced at the 5mC rich regions or at least in close proximity to this site.

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