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Effect of VP-16-213 on the Intracellular Degradation of DNA in HeLa Cells†

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ABSTRACT: The effect of VP-16-213 on cellular DNA was studied by following the sedimentation profiles of radioactive DNA in HeLa cells on alkaline sucrose gradients. In VP-16-213 treated cells, high-molecular-weight DNA is converted to a lower molecular-weight form in a dose-dependent, temperature-dependent reaction. The effect of VP-16-213 on cellular DNA is reversed after the drug has been removed from the growth medium for 150 min. These results suggest that VP-16-213 induces single-stranded breaks in DNA in HeLa cells and that HeLa cells can repair these breaks within 150 min. The nonglucoside derivative of VP-16-213, 4′-demethylepipodophyllotoxin, also induces the cleavage of cellular DNA but podophyllotoxin has no effect on DNA. A structure-activity relationship study, in which the effects of various VP-16-213 and podophyllotoxin congeners were tested for their ability to cleave cellular DNA, revealed that an hydroxyl group at the C-4′ position is required for activity and that the configuration of the C-4 carbon influences the activity of a congener. These results may offer insights into the mechanism of action of VP-16-213 as an antitumor agent.

Podophyllotoxin is a cytotoxic agent which arrests eukaryotic cells in metaphase by inhibiting microtubule formation in the mitotic spindle apparatus (Wilson et al., 1974). Unlike other compounds, such as vinblastine and vincristine which exhibit a similar mechanism of action and are important cancer chemotherapeutic agents, podophyllotoxin has little potential as an antitumor drug because of its severe toxicity in man. Stähelin (1969, 1970, 1972) has chemically modified different positions of the podophyllotoxin ring system in an attempt to discover a less toxic, more water-soluble antitumor derivative. 4′-Demethylepipodophyllotoxin ethylidene-β-D-glucoside (VP-16-213) and 4′-demethylepipodophyllotoxin thenylidene-β-D-glucoside (VM-26) are two semisynthetic podophyllotoxin derivatives (Figure 1) which have demonstrated antitumor activity in clinical studies.

These derivatives differ from podophyllotoxin in their mode of action. Unlike podophyllotoxin, which inhibits cells in metaphase, VP-16-213 arrests cells in a premitotic stage of the cell cycle (Stähelin, 1972; Grieder et al., 1974). Treatment of murine mastocytoma (P-815X2) cells with VP-16-213 results in the inhibition of cell multiplication and of thymidine incorporation into acid-insoluble material, and reduction of the number of cells in mitosis (Grieder et al., 1974). Huang et al. (1973) noted a high incidence of chromosomal aberrations in human hematopoietic cell lines after incubation with VP-16-213.

Our previous studies with VP-16-213 in HeLa cells demonstrated that VP-16-213 is an inhibitor of nucleoside transport, an activity it shares with podophyllotoxin. In contrast to podophyllotoxin, which inhibits microtubule assembly in vitro and in vivo (Wilson et al., 1974), VP-16-213 does not inhibit microtubule assembly in vitro (Loike and Horwitz, 1976). The present communication describes the effects of VP-16-213 on DNA in HeLa cells and examines the action of several podophyllotoxin and VP-16-213 congeners in an attempt to explain the relationship between chemical and biological activity.

Materials and Methods

Materials. 4′-Demethylepipodophyllotoxin, 4′-demethylpodophyllotoxin, VP-16-213, and 4′-demethyldeoxypodophyllotoxin were kindly provided by Sandoz, Switzerland. Podophyllotoxin, picropodophyllotoxin, VM-26, deoxypodophyllotoxin, α- and β-peltatin were obtained from the National Cancer Institute. Epipodophyllotoxin was a gift from Dr. F. Johnson. [^{3}H]Thymidine (6.7 Ci/mmol) and scintillation fluid (Formula 950) were purchased from New England Nuclear. GF/C glass filters (2.5 cm) were purchased from Whatman; thymidine from Sigma; culture media, L-glutamine, and serum from Grand Island Biological Co.

Cultures. Monolayer and spinner cultures of HeLa cells were maintained as previously described (Loike and Horwitz, 1976).

Preparation of Radioactive HeLa Cell and Adenovirus DNA. Twenty milliliters of HeLa cells at a density of 4×10^5 cells/ml were incubated with 2.5 μCi of [^{3}H]thymidine at 37 °C. After 60 min, the suspension was diluted to 100 ml with cold minimal essential medium containing 20 μM [^{12}C]thymidine, washed with 50 ml of the same solution, and resuspended at a density of 4×10^5 cells/ml in minimal essential

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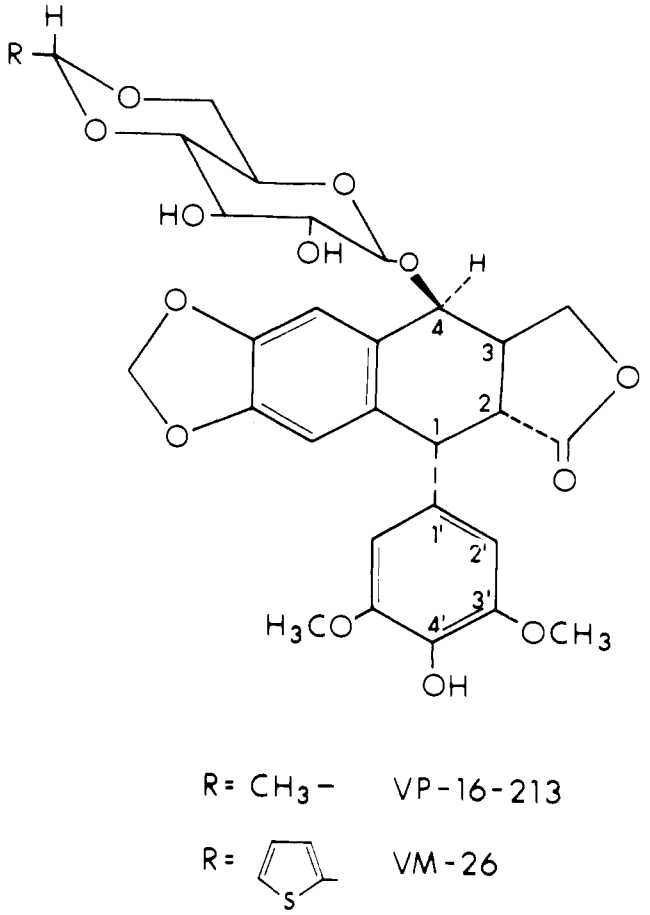


FIGURE 1: Structural formulas of VP-16-213 and VM-26.

medium, supplemented with 5% fetal calf serum and 1% glutamine.

Type 2 adenovirus was grown and its DNA was purified as previously described by Horwitz (1971).

Alkaline Sucrose Gradients. HeLa Cells. [^{3}H]Thymidine-labeled cells at a density of 4×10^5 cells/ml were collected by centrifugation, resuspended in 0.5 ml of 0.15 M NaCl, and gently layered over a 15-ml 5–20% linear alkaline sucrose gradient. Gradients were prepared as described by Horwitz et al. (1971). They were made on a 0.5-ml cushion of CsCl (density 1.8 g/ml) in a 17-ml cellulose nitrate tube and overlaid with a 0.5-ml lysing layer containing 1.0 M NaCl, 0.19 M NaOH, 0.01 M EDTA,[1] and 0.5% sodium deoxycholate. The cells were placed on top of the lysing layer and centrifuged in a Spinco SW 27 rotor at 30 000*g* for 16 h at 4 °C. Fractions, 0.6–0.7 ml, were collected by means of a finger pump starting at 1.5 cm above the bottom of the tube. The solution remaining at the bottom of the tube was disrupted by sonic oscillation for 20 s in a Branson model W 185 sonifier set at position 6. All fractions were adjusted to a final trichloroacetic acid concentration of 12.5% and remained at 2 °C for 15 min before collection of the precipitates on glass filters. Radioactivity was determined as described below.

Adenovirus DNA. A 0.01-ml incubation mixture containing purified adenovirus DNA (2000 cpm) in Tris-HCl buffer, pH 8.1, was layered over an 11.0-ml 5–20% alkaline sucrose gradient prepared in a 12-ml cellulose nitrate tube and centrifuged in a SW 41 rotor at 184 000*g* for 5 h at 4 °C. Fractions, 0.4–0.5 ml, were collected and precipitated as described for HeLa DNA.

Radioactivity Determination. Radioactivity was determined in a Packard Tri-Carb Liquid Scintillation counter using 8 ml of Formula 950 as the scintillation fluid.

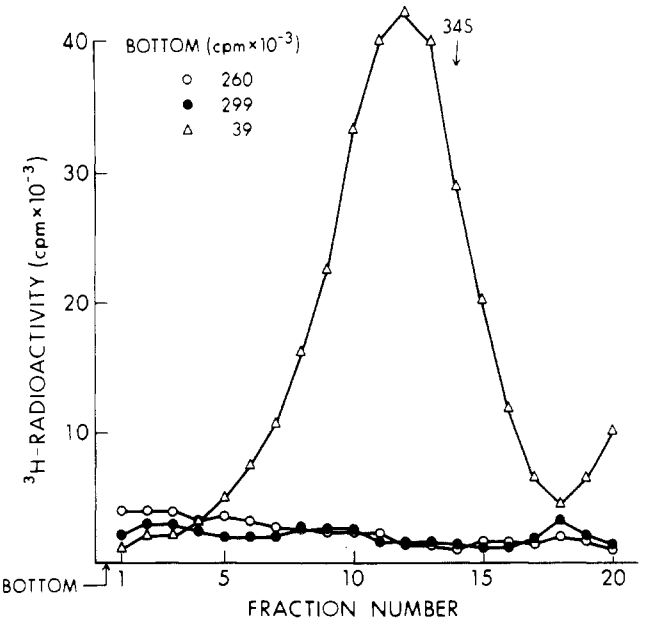


FIGURE 2: Sedimentation of DNA from VP-16-213 and podophyllotoxin-treated cells. HeLa cells, containing labeled DNA, were incubated at 37 °C in the presence or absence of drug. After 60 min, the cells were analyzed by alkaline sucrose gradient centrifugation as described under Materials and Methods. "Bottom" refers to the lower 1.5 cm of the gradient tube. Purified type 2 adenovirus, containing [^{14}C]thymidine-labeled DNA, sediments at 34 S and was used as a marker. No additions, (○); 100 μM podophyllotoxin (●); 100 μM VP-16-213 (△).

Results

Effects of VP-16-213 and Podophyllotoxin on DNA in HeLa Cells. The effects of podophyllotoxin and VP-16-213 on radioactive DNA in HeLa cells were monitored by following the sedimentation of cellular DNA on alkaline sucrose gradients. HeLa cells, prelabeled with [^{3}H]thymidine, were incubated at 37 °C for 1 h in the presence or absence of drug before placing the cells on the gradient. Radioactive DNA from untreated HeLa cells or cells treated with 100 μM podophyllotoxin appeared as acid-insoluble material of high molecular weight at the bottom of the gradient (Figure 2). In cells treated with 100 μM VP-16-213, the high-molecular-weight DNA was converted to a lower molecular-weight form and appeared in the middle of the gradient. This change in sedimentation is interpreted to be a result of single-stranded breaks in DNA; the size of these DNA fragments was approximately 40 S. The results in Figure 3 demonstrate that the fragmentation of DNA induced by VP-16-213 is dose dependent. Even when VP-16-213 was present at a concentration of 1 μM, an alteration in the sedimentation of labeled cellular DNA was observed.

The effect of VP-16-213 on DNA in HeLa cells was examined at various time intervals after exposing cells to VP-16-213. During a 1-min incubation period, VP-16-213 induced a partial conversion of high-molecular-weight cellular DNA to a lower molecular-weight form. At 30 min the extent of fragmentation was the same as after a 150-min incubation period. HeLa cells were also incubated in the presence of VP-16-213 for 1 h at different temperatures and the DNA was analyzed by alkaline sucrose gradient centrifugation. At 2 °C,

[1] Abbreviations used are: EDTA, (ethylenedinitrilo)tetraacetic acid; SAR, structure-activity relationship.

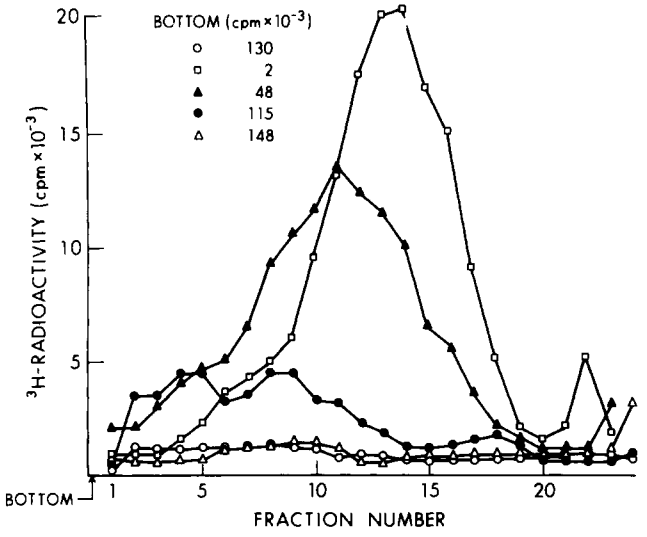


FIGURE 3: Effect of various concentrations of VP-16-213 on the sedimentation of DNA from HeLa cells. HeLa cells, containing labeled DNA, were incubated at 37 °C in the presence or absence of various concentrations of VP-16-213. After 60 min, the cells were analyzed by alkaline sucrose gradient centrifugation as described under Materials and Methods. No additions (○); 100 μM VP-16-213 (□); 10 μM VP-16-213 (▲); 1 μM VP-16-213 (●); 0.1 μM VP-16-213 (△).

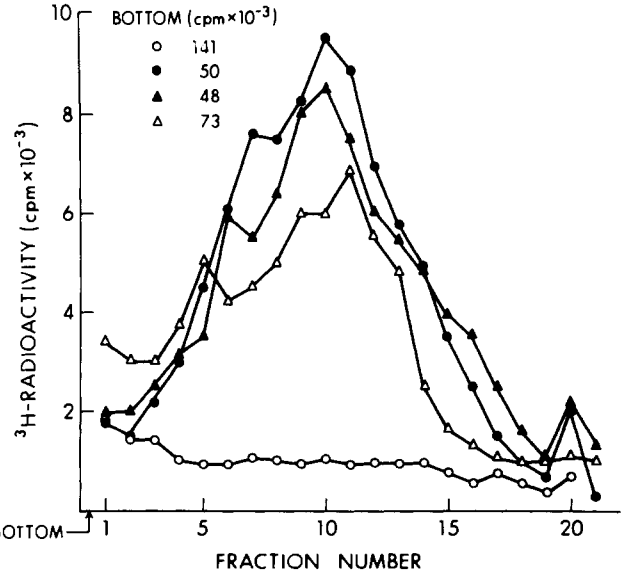


FIGURE 4: Reversal of the effect of VP-16-213 on the sedimentation of DNA from HeLa cells. HeLa cells, containing labeled DNA, were incubated at 37 °C in the presence or absence of 10 μM VP-16-213. After 30 min, the cells were washed three times with complete medium and resuspended in complete medium plus or minus VP-16-213. After the second incubation period (30 or 150 min), cells were analyzed by alkaline sucrose gradient centrifugation as described under Materials and Methods. The first and second incubations contained, respectively: (●) 10 and 10 μM, at 30 min; (▲) 10 and 10 μM, at 150 min; (△) 10 μM and no additions, at 30 min; (○) 10 μM or no additions and no additions, at 150 min.

VP-16-213 induced partial conversion of cellular DNA to the lower molecular-weight form. At 18 °C fragmentation of HeLa cell DNA by VP-16-213 was as extensive as when the incubation was at 37 °C.

Reversal of DNA Fragmentation. The possibility that HeLa cells could repair VP-16-213 induced breaks was examined by washing VP-16-213 treated cells and resuspending them in drug-free medium. After various time intervals, the DNA was analyzed by alkaline sucrose gradient centrifugation. HeLa cells containing labeled DNA were incubated for 30 min in the presence or absence of 10 μM VP-16-213, washed three times with complete medium, and incubated for either 30 or 150 min in complete medium plus or minus 10 μM VP-16-213. Cells incubated for 30 min in the presence of VP-16-213, washed, and resuspended in the absence of drug for an additional 30 min showed significant quantities of DNA at the 40S region of the gradient. However, if cells were allowed to remain in drug-free medium for 150 min, no radioactive peak was seen at the 40S region and the DNA sedimented as high-molecular-weight DNA (Figure 4). These results suggest that, within 150 min, HeLa cells were able to repair the DNA fragmentation induced by VP-16-213.

The effect of temperature on repair of VP-16-213 induced breaks in DNA was studied. Cells were treated with 10 μM VP-16-213 for 30 min, washed with drug-free medium, and incubated for an additional 150 min at either 2 or 37 °C. At 37 °C the cells were able to repair VP-16-213 induced breaks; the DNA from these cells sedimented as high-molecular-weight species. The DNA from cells incubated at 2 °C failed to repair VP-16-213 induced breaks and remained at the 40S region in the sucrose gradient.

Effects of Podophyllotoxin Congeners on HeLa Cell DNA and Nucleoside Uptake. A variety of podophyllotoxin congeners were tested for their ability to induce the conversion of high-molecular-weight cellular DNA to a lower molecular-weight form. Table I and Figure 5 summarize the results of our experiments. At 100 μM, VP-16-213, VM-26, and 4′-demethylepipodophyllotoxin were able to convert cellular DNA to a lower molecular-weight form. 4′-Demethylpodophyllotoxin, 4′-demethyldeoxypodophyllotoxin, and α-peltatin were less active in cleaving DNA, while podophyllotoxin, epipodophyllotoxin, deoxypodophyllotoxin, and β-peltatin were inactive.

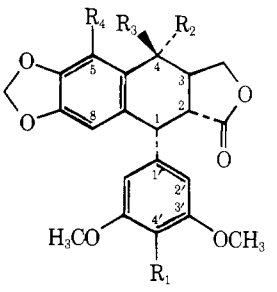
Each congener was also tested for its ability to inhibit thymidine and uridine uptake into HeLa cells. In Table I, the concentration of drug necessary to inhibit, by 50%, the uptake of thymidine and uridine into HeLa cells is given. Deoxypodophyllotoxin and epipodophyllotoxin were the most potent inhibitors of thymidine and uridine uptake. Podophyllotoxin, 4′-demethylepipodophyllotoxin, and α- and β-peltatin demonstrated intermediate activity and 4′-demethylpodophyllotoxin, VP-16-213, and VM-26 were the least active inhibitors.

Effects of VP-16-213 on Purified Type 2 Adenovirus DNA. The effect of VP-16-213 on ^{14}C-labeled adenovirus DNA was examined by alkaline sucrose gradient centrifugation (Figure 6). Purified adenovirus DNA was incubated for 1 h at 37 °C in the presence or absence of 100 μM VP-16-213 in Tris-HCl buffer, pH 8.1, containing 10 mM 2-mercaptoethanol. No difference between VP-16-213 treated and untreated DNA was observed in alkaline sucrose gradient profiles. Bleomycin, a polypeptide which produces single-stranded breaks in a variety of DNAs, served as a control (Suzuki et al., 1969). When adenovirus DNA was incubated in the presence of 66 μM bleomycin and 10 mM 2-mercaptoethanol, the DNA, which has a normal sedimentation constant of 34 S (Green et al., 1967), sedimented as a lower molecular-weight fragment at the top of the gradient. These results indicate that VP-16-213, in contrast to bleomycin, does not induce single-stranded breaks in purified type 2 adenovirus DNA.

Discussion

Alkaline sucrose gradient sedimentation profiles of radioactive DNA in HeLa cells have been utilized to follow the

TABLE I.



Congener	R_1	R_2	R_3	R_4	% Low-Mol-Weight Form DNA[a]	Drug Concn (μM)[b] Thymidine	Uridine
Podophyllotoxin	OCH_3	OH	H	H	5	9	5
4′-Demethylpodophyllotoxin	OH	OH	H	H	33[c]	40	40
Epipodophyllotoxin	OCH_3	H	OH	H	3	0.8	5
4′-Demethylepipodophyllotoxin	OH	H	OH	H	68[c]	2	10
VP-16-213	OH	H	Ethylidene glucoside	H	75[c]	25	20
VM-26	OH	H	Thenylidene glucoside	H	83[c]	45	20
Deoxypodophyllotoxin	OCH_3	H	H	H	5	0.7	0.5
4′-Demethyldeoxypodophyllotoxin	OH	H	H	H	56[c]		
β-Peltatin	OCH_3	H	H	OH	5	10	10
α-Peltatin	OH	H	H	OH	40[c]	8	10

[a] HeLa cells (4×10^5 cells/ml) containing labeled DNA were incubated at 37 °C for 1 h in the presence or absence of 100 μM drug. The cells were analyzed by alkaline sucrose gradient centrifugation as described under Materials and Methods. Values are expressed as the percent of radioactivity present as lower molecular-weight species, which corresponds to the DNA present in the entire gradient, except for the 1.5 cm CsCl cushion at the bottom. In untreated cells, 15% of the labeled DNA was found throughout the gradient above the CsCl cushion. These counts were subtracted from all values. [b] The uptake of thymidine and uridine into HeLa cells grown as monolayers was measured as previously described (Loike and Horwitz, 1976). Values represent the concentration of drug necessary to inhibit the uptake of thymidine or uridine into the cell by 50%. [c] Values were obtained from sedimentation profiles seen in Figure 5.

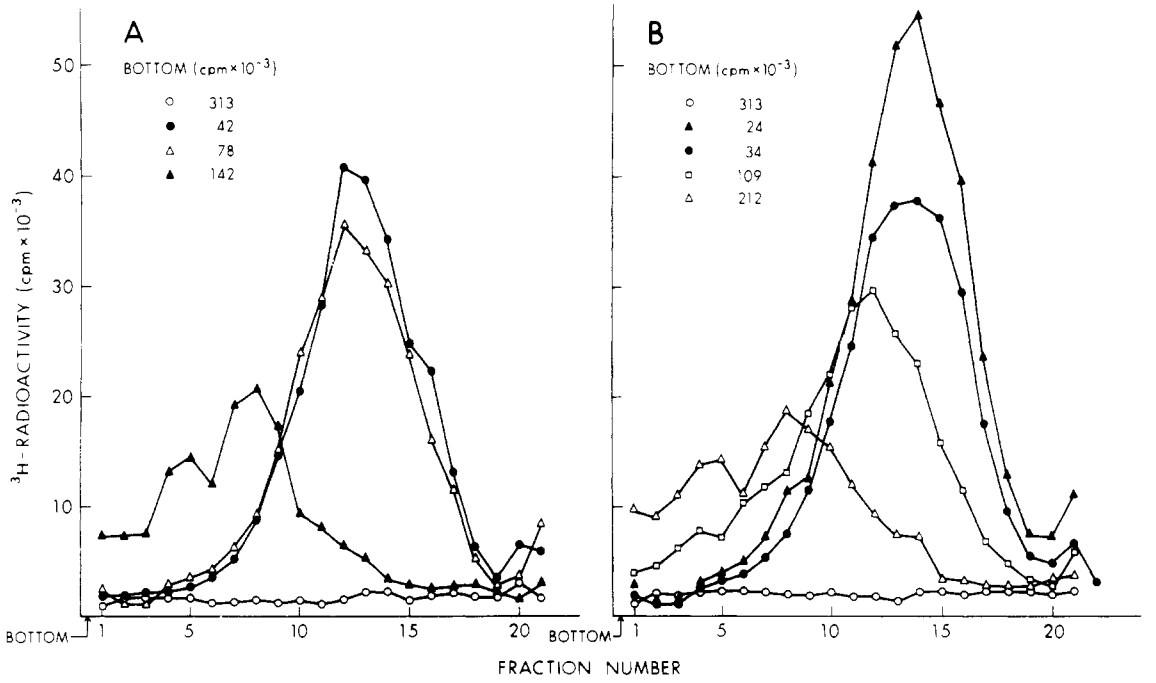


FIGURE 5: Effect of VP-16-213 congeners on the sedimentation of DNA in HeLa cells. HeLa cells, containing labeled DNA were incubated at 37 °C in the presence or absence of 100 μM drug. After 60 min, the cells were analyzed by alkaline sucrose gradient centrifugation as described under Materials and Methods. (A) No additions (○); VP-16-213 (●); 4′-demethylepipodophyllotoxin (△); α-peltatin (▲). (B) No additions (○); VM-26 (▲); VP-16-213 (●); 4′-demethyldeoxypodophyllotoxin (□); 4-demethylpodophyllotoxin (△).

effect of VP-16-213 on cellular DNA. In VP-16-213 treated cells, high-molecular-weight DNA is converted to a lower molecular-weight form. The effect of VP-16-213 on cellular DNA is dose and temperature dependent and reversible after the drug has been removed from the culture media for 150 min. We interpret the change in sedimentation of DNA to be a result of DNA breaks induced by VP-16-213. Our results indicate that HeLa cells can repair VP-16-213 induced breaks and,

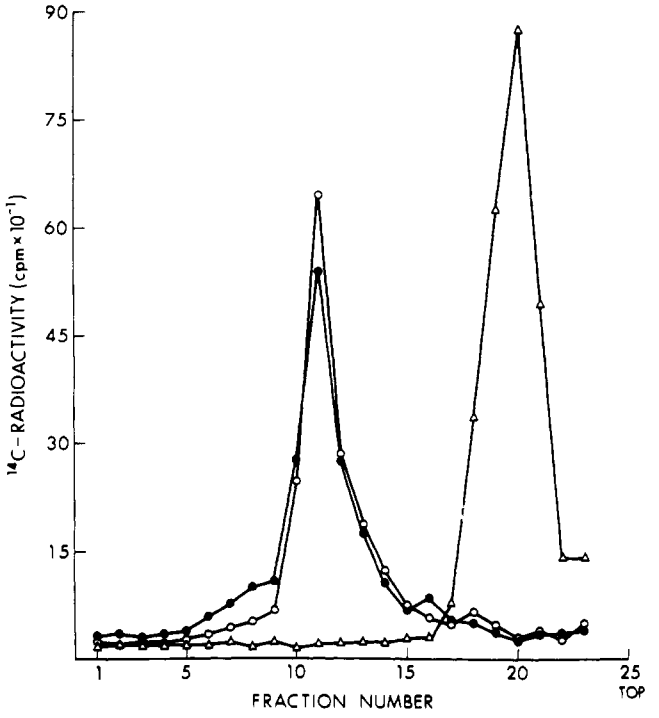


FIGURE 6: Effect of VP-16-213 on purified type 2 adenovirus DNA. A 0.01-ml incubation mixture containing 50 mM Tris-HCl, pH 8.1, 10 mM 2-mercaptoethanol, and purified type 2 adenovirus DNA was incubated at 37 °C in the presence or absence of drug. After 60 min, the mixture was layered on an alkaline sucrose gradient and centrifuged as described under Materials and Methods. No additions (○); 100 μM VP-16-213 (●); 66 μM bleomycin (△).

therefore, suggest that these breaks are single stranded, since double-stranded breaks are not normally repaired. At 2 °C, a temperature at which DNA synthesis does not take place, there is no repair of VP-16-213 induced breaks in HeLa cell DNA.

VP-16-213 does not cleave purified HeLa cell DNA (unpublished data) or adenovirus DNA, indicating that the effect of VP-16-213 on DNA is not a result of a direct chemical cleavage by VP-16-213 on DNA. The action of VP-16-213 has features which are similar to camptothecin (Horwitz et al., 1971); both drugs break DNA in HeLa cells but fail to cleave purified DNA. These effects are in contrast to the action of bleomycin (Suzuki et al., 1969) or neocarzinostatin (Beerman and Goldberg, 1974), which cleave DNA in vivo as well as in vitro.

If VP-16-213 does not act directly on DNA in HeLa cells, several possible mechanisms could explain its mode of action. VP-16-213 may induce breaks in DNA by activating one or several cellular endonucleases. Alternatively, the cell may metabolize VP-16-213 to a product which could directly cleave DNA or induce cellular endonucleases. Creaven et al. (1975) have recently reported that 40% of tritium-labeled VP-16-213 recovered in the urine of cancer patients was in the form of VP-16-213 metabolites. 4′-Demethylepipodophyllic acid glucoside was identified as the major urinary metabolite of VP-16-213 in man (Allen et al., 1976). The effects of VP-16-213 may result from the interaction of the drug or its metabolites with chromatin, thereby altering the properties of chromatin as a substrate for cellular endonucleases.

It is difficult to exclude the possibility that the single-stranded breaks induced by VP-16-213 are a result of alkali-labile bonds. Such bonds could be created by an interaction of VP-16-213 and cellular DNA, and appear as breaks when cells are exposed to alkaline conditions on gradients. However, Huang et al. (1973) have reported that human hematopoietic cell lines treated with VP-16-213 contain a high incidence of chromosomal aberrations during metaphase. Such observations support our hypothesis that VP-16-213 induced breaks in DNA are not a result of alkali-labile bonds.

In contrast to VP-16-213, podophyllotoxin does not induce breaks in cellular DNA. We have examined several podophyllotoxin congeners in order to understand the structure-activity relationship (SAR) for DNA cleavage. Our SAR study reveals that 4′-demethylepipodophyllotoxin, the nonglucoside form of VP-16-213, and VM-26, are as active as VP-16-213 in fragmenting DNA in HeLa cells. VM-26, which differs from VP-16-213 in the glucoside moiety, demonstrates greater activity in inducing the cleavage of DNA than VP-16-213. Differences in activities between VP-16-213 and VM-26 may be a result of different permeability rates across the cell membrane or different metabolic rates (if these drugs are metabolized). The SAR study has also demonstrated that VP-16-213 congeners are active in fragmenting DNA if the 4′-carbon contains an hydroxyl group. Replacing the 4′-hydroxyl with a methoxy group, as in podophyllotoxin, renders a compound inactive. The configuration of the C-4 carbon affects the potency of the 4′-demethyl congeners such that the "epi" isomer (*S* configuration) demonstrates greater activity than its optical isomer in which the ligands at C-4 are in *R* configuration. 4′-Demethyldeoxypodophyllotoxin lacks an OH group at its C-4 carbon and demonstrates intermediate activity in cleaving DNA (as compared to the *R* and *S* isomers). β-Peltatin differs from podophyllotoxin at two sites, the presence of an hydroxyl group at C-5 carbon and two hydrogens at C-4 carbon (Table I). β-Peltatin, like podophyllotoxin, does not fragment DNA in HeLa cells. α-Peltatin, the 4′-demethyl congener of β-peltatin, does induce the cleavage of DNA, thereby supporting our hypothesis that a 4′-hydroxyl group must be present for activity.

The effects of these congeners on thymidine and uridine uptake into HeLa cells were also examined (Table I). Each of these congeners inhibited nucleoside uptake but exhibited different potencies. With the limited number of congeners available, it is difficult to determine which structural moieties of podophyllotoxin or VP-16-213 are responsible for the inhibition of thymidine and uridine uptake. Our SAR study does suggest that compounds containing a C-4′-methoxy group are more potent inhibitors of transport than the corresponding compounds containing a 4′-hydroxyl group. Furthermore, our results indicate that the effect of VP-16-213, VM-26, and the 4′-demethyl podophyllotoxin congeners on the breakage of DNA is unrelated to their effect on nucleoside uptake. Several podophyllotoxin congeners (e.g., podophyllotoxin, epipodophyllotoxin, and β-peltatin) inhibit nucleoside uptake into HeLa cells but fail to demonstrate any effect on the fragmentation of DNA in our system.

VP-16-213 does not inhibit microtubule assembly in vitro; however, it does inhibit nucleoside transport and induces single-stranded breaks in DNA in HeLa cells. The nonglucoside derivative of VP-16-213, 4′-demethylepipodophyllotoxin, also inhibits nucleoside transport and induces breaks in DNA, but, unlike VP-16-213, is a potent inhibitor of microtubule assembly in vitro (Loike and Horwitz, 1976). Although cellular glucosidases could cleave the glucoside moiety of VP-16-213 yielding 4′-demethylepipodophyllotoxin, which could act at several intracellular sites, the evidence suggests that 4′-demethylepipodophyllotoxin does not accumulate in cells. Cell-cycle studies (Stähelin, 1972; Grieder et al., 1974; Krishan et

al., 1975) have demonstrated that VP-16-213 arrests cells in a different phase of the cell cycle than that of podophyllotoxin. While agents like podophyllotoxin, which inhibit microtubule assembly, arrest cells during M phase, VP-16-213 treated cells are arrested in a premitotic (S or G_2) phase of the cell cycle. Other investigators (Huang et al., 1973) have reported that VP-16-213 causes chromosomal aberrations in cultured cells and arrests cells in S or G_2 phase. Such results suggest that the glucoside moiety of VP-16-213 is not removed, since VP-16-213 treated eells are not arrested in metaphase. These cell cycle studies, coupled with our observations, support the idea that a major action of VP-16-213 in cells is related to its DNA effect.

In conclusion, our results offer possible insights into the mechanism of action of VP-16-213 and indicate that minor chemical modifications of a parent compound can yield derivatives which not only have different potencies, but also possess different biological properties. Podohyllotoxin, VP-16-213, and their congeners offer an excellent group of drugs for studying the relationship between structural alterations and biological properties. The fact that VP-16-213 is not a mitotic inhibitor is consistent with current evidence (Dombernowsky et al., 1976) that patients treated with VP-16-213 do not become cross resistant to vincristine. Thus, combination chemotherapy of VP-16-213 and a mitotic inhibitor (vincristine and vinblastine) would now merit serious consideration.

Acknowledgments

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