Lang, A. R. G. (1967), *Aust. J. Chem. 20,* 2017.
Leifer, L., and Högfeldt, E., (1964), in First Australian Conference on Electrochemistry, Friend, A., and Gutmann, F., Ed., Oxford, Pergamon, p 107.
Leifer, L., and Högfeldt, E. (1973), *Acta Chem. Scand. 27,* 4007.
Levine, Y. K., Birdsall, N. J. M., Lee, A. G., and Metcalfe, J. C. (1972), *Biochemistry 11,* 1416.
Ludemann, H. D. (1972), *Z. Naturforsch. 27,* 1196.
Ringler, D. G., and Anver, M. R. (1975), *Science 188,* 166.
Sheetz, M. P., and Chan, S. I. (1972), *Biochemistry 11,* 4573.
Ts'o, P. O. P., and Chan, S. I. (1964), *J. Am. Chem. Soc. 86,* 4176.
Ts'o, P. O. P. Melvin, I. S., and Olson, A. C. (1963), *J. Am. Chem. Soc. 85,* 1289.
Wen, W. Y., Saito, S., and Lee, C. M. (1966), *J. Phys. Chem. 70,* 1244.

Effects of Podophyllotoxin and VP-16-213 on Microtubule Assembly in Vitro and Nucleoside Transport in HeLa Cells†

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ABSTRACT: VP-16-213, a semisynthetic derivative of podophyllotoxin, is an active antitumor agent. In this paper, the effects of VP-16-213 and podophyllotoxin on microtubule assembly in vitro and nucleoside transport in HeLa cells are compared. At 100 μM, VP-16-213 does not inhibit microtubule assembly in vitro, while 5 μM podophyllotoxin completely prevents the formation of microtubules. The presence of the glucoside moiety in VP-16-213 is responsible for the inactivity of VP-16-213 in this system because 4′-demethylepipodophyllotoxin, the nonglucoside congener of VP-16-213, inhibits microtubule assembly. In HeLa cells, VP-16-213 and podophyllotoxin share a common biological property; both agents inhibit the uptake of thymidine and uridine into cells by inhibiting the facilitated diffusional component of nucleoside transport. The concentrations of drug necessary to inhibit thymidine and uridine uptake into HeLa cells by 50% are 10 and 5 μM, respectively, for podophyllotoxin, and 25 and 20 μM for VP-16-213. The action of podophyllotoxin on nucleoside transport appears unrelated to its effect on microtubule assembly, since VP-16-213, which does not inhibit microtubule assembly, inhibits nucleoside transport.

Podophyllum resin is a crude extract isolated from the roots and rhizomes of the plants of the podophyllum species. It has been used in man as a cathartic, anthelminthic agent and a remedy for condyloma acuminatum (Kelly and Hartwell, 1954; Hartwell and Schrecker, 1958). The active constituent of this resin is podophyllotoxin, a cytotoxic compound whose mechanism of action involves at least two cellular processes, cell mitosis and nucleoside transport. During cell mitosis, spindle fibers composed of microtubules separate the duplicated chromosomes to poles located at opposite ends of the dividing cell. At low concentrations (1 μM), podophyllotoxin binds to the microtubule subunit, tubulin, inhibiting its polymerization into microtubules and arresting cell division in mitosis (Wilson et al., 1974). At higher concentrations (10–100 μM), Mizel and Wilson (1972) have shown that podophyllotoxin inhibits nucleoside transport.

Over the last decade many investigators have synthesized or isolated compounds related to podophyllotoxin in search of clinically effective antitumor agents. Recently, a semisynthetic podophyllotoxin derivative, 4′-demethylepipodophyllotoxin ethylidene-β-D-glucoside, VP-16-213, was synthesized by Stähelin (1973) and has activity against certain solid tumors and leukemias (Dombernowsky et al., 1972; Nissen et al., 1972; Creaven et al., 1975). VP-16-213 differs from podophyllotoxin at three positions: (1) VP-16-213 contains a glucoside moiety at the C-4 carbon, (2) it has enantiomeric configuration of podophyllotoxin at C-4 carbon atom, and (3) VP-16-213 contains a hydroxyl group at the C-4′ position (Figure 1). In contrast to podophyllotoxin, which blocks cells in mitosis, VP-16-213 has been reported to block L-1210 cells in a premitotic stage of the cell cycle (Grieder et al., 1974). VP-16-213 has also been shown to cause a high incidence of chromosomal aberrations in human hematopoietic cell lines (Huang et al., 1973).

In the present paper, we have examined the effects of podophyllotoxin and VP-16-213 on microtubule assembly in vitro and report that, in contrast to podophyllotoxin, VP-16-213 does not inhibit the in vitro polymerization of tubulin. We have also compared the effects of VP-16-213 and podophyllotoxin on macromolecular synthesis and nucleoside transport in HeLa cells, and found that both drugs are effective inhibitors of nucleoside transport. VP-16-213 also induces single-stranded breaks in HeLa cell DNA, an effect not seen in podophyllotoxin-treated cells. The characterization of this effect on DNA is presented in the following paper (Loike and Horwitz, 1976).

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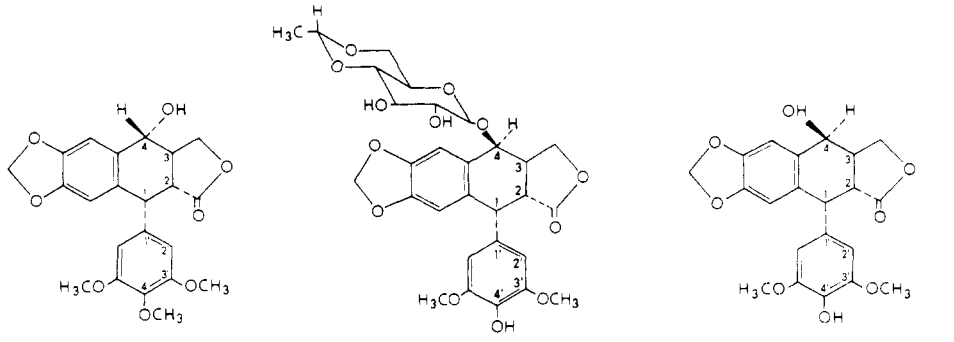

FIGURE 1: Structural formulas of podophyllotoxin, VP-16-213 and 4'-demethylepipodophyllotoxin.

Materials and Methods

Materials. Podophyllotoxin was obtained from the National Cancer Institute. VP-16-213 and 4'-demethylepipodophyllotoxin were kindly provided by Sandoz, Switzerland. Each drug consisted of a single component, as analyzed by thin-layer chromatography. All compounds were dissolved in dimethyl sulfoxide at a concentration of 0.1 M and stored at −20 °C. The final concentration of dimethyl sulfoxide used in each experiment was less than 1%, a concentration that had no effect on control reactions. Fetal calf serum, tissue culture media, and trypsin were purchased from Grand Island Biological Co.; Triton X-100 from Rugar Chemical Co.; NCS tissue solubilizer from Amersham and Searle; and glass filters (GF/C, 2.5 cm) from Whatman. Uniformly labeled [^{14}C]leucine (252 mCi/mmol), [2-^{14}C]uridine (57 mCi/mmol), [5-^{3}H]uridine (29.65 Ci/mmol), [2-^{14}C]thymidine (57 mCi/mmol), [methyl-^{3}H]thymidine (6.7 Ci/mmol), [8-^{14}C]adenosine (51.2 Ci/mmol), [^{3}H]adenosine (31.2 Ci/mmol), 2-deoxy-D-[1-^{14}C]glucose (47 mCi/mmol), [8-^{3}H]guanosine (15 Ci/mmol), [^{3}H]choline (4.2 Ci/mmol), and Aquasol were purchased from New England Nuclear. Glass scintillation vials for growing cells in monolayer culture were obtained from Packard and autoclavable caps for the vials from A. H. Thomas Co. Norit-211 activated charcoal was purchased from Eastman Chemical Co.

Preparation of Tubulin. Tubulin was purified from fresh chicken brains by the assembly-disassembly procedure described by Shelanski et al. (1973) and stored at −20 °C in Mes[1] buffer (0.1 M, 2-(*N*-morpholino)ethanesulfonic acid, 1 mM EGTA, and 0.5 mM $MgCl_2 \cdot 6H_2O$, pH 6.6) containing 4 M glycerol. Prior to each experiment, the stored tubulin was dialyzed for 3 h at 4 °C against 100 volumes of Mes buffer and centrifuged at 120 000*g* for 30 min at 4 °C. The supernatant containing the tubulin was maintained at 4 °C in the presence of 1 mM GTP. Tubulin purity was greater than 80%, as determined by staining with Coomassie blue after electrophoresis on 5 or 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate and 8 M urea (Feit et al., 1971). Protein concentration was determined by the method of Lowry et al. (1951). Microtubule assembly was monitored by following changes in turbidity (Gaskin et al., 1974) at 350 nm on a Gilford 2400 spectrophotometer equipped with an automatic recorder and a Haake thermostatically regulated liquid circulator to maintain temperature.

Electron Microscopy. All tubulin samples were monitored for microtubule formation by electron microscopy. At the completion of the assembly reaction, one drop of sample was applied to a formvar grid for 1 min and stained by rinsing with six drops of 1% uranyl acetate. The grid was dried by gentle blotting with filter paper and observed at 6000× and 65 000× with a Siemens, Elmiskop 1A electron microscope.

Incorporation of Macromolecular Precursors into Acid-Insoluble Material. HeLa cells, S_3, were grown in spinner culture in Joklik's modified Eagle's minimal essential medium (Mem) supplemented with 5% fetal calf serum and 1% glutamine (complete Mem). The incorporation of radioactive thymidine, uridine, and leucine into trichloroacetic acid-insoluble material in HeLa cells was measured as previously described (Horwitz et al., 1971).

Nucleoside Uptake Experiments in HeLa Cells Grown in Monolayer Cultures. Incorporation of labeled precursors into Cl_3CCOOH-soluble and -insoluble material was assayed with minor modifications by the procedure of Klevecz and Stubblefield (1967). HeLa cells, cloned by J. Williams and provided by M. Horwitz, were grown in a 5% CO_2 atmosphere as monolayers in 75 cm^2 Falcon flasks in Eagle's Mem, supplemented with 10% fetal calf serum and 1% glutamine. Approximately 15 h before an experiment, the cells were trypsinized with 0.25% trypsin and transferred to sterile glass scintillation vials at a density of 10^5 cells/vial. At the start of the uptake experiment, the growth medium was gently aspirated and 1 ml of fresh medium containing 1.5 mM Hepes buffer, to insure proper pH, was added to the vials. Serum was omitted in order to eliminate nucleosides which may be present in serum (Forsdyke, 1971). After a 1-h incubation at 37 °C, the medium was aspirated and fresh medium of identical composition containing labeled nucleosides with or without drug was added. At the end of the specified incubation interval at 37 °C, the vials were placed at 2 °C, the medium was immediately removed by aspiration, and the cells were washed three times with cold basal salt solution (BSS) (Plagemann and Roth, 1969). One milliliter of 10% Cl_3CCOOH was added to the vials and the cells were chilled for 2 h at 2 °C. The radioactivity in an aliquot of supernatant (Cl_3CCOOH-soluble material) was determined as described below. The Cl_3CCOOH-insoluble layer was washed once with cold 10% Cl_3CCOOH and solubilized with 1 ml of NCS tissue solubilizer at 37 °C for 2 h.

[1] Abbreviations used are: Mes, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, [ethylenebis(oxoethylenenitrilo)]tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PPO, 2,5-diphenyloxazole; GTP, guanosine triphosphate; UMP, UDP, UTP, uridine mono-, di-, and triphosphates; TMP, TDP, TTP, thymidine mono-, di-, and triphosphates; TLC, thin-layer chromatography.

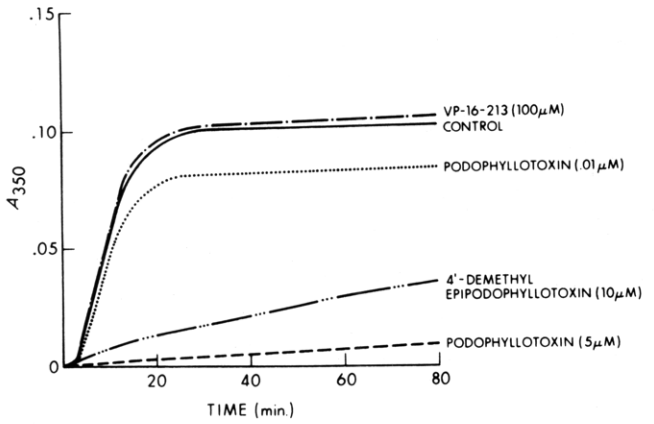

FIGURE 2: Effects of podophyllotoxin and VP-16-213 on microtubule assembly. Tubulin was isolated from chicken brains as described under Materials and Methods. Cuvettes containing Mes buffer, plus or minus drug, were maintained at room temperature and shifted to 37 °C only after the addition of 1.0 mg/ml of tubulin and 1 mM GTP. Data points were taken at 1-min intervals.

2-Deoxy-D-Glucose, Choline, and Leucine Uptake Experiments. HeLa cells grown in monolayer cultures in sterile glass vials were prepared as described above. At the start of the experiment, cells were incubated at 37 °C with (a) Mem containing 1.5 mM Hepes for 2-deoxy-D-glucose and choline uptake studies, or (b) leucine-depleted Mem containing 1.5 mM Hepes for leucine uptake studies. After 1 h, the medium was aspirated and fresh Mem of the same composition containing labeled leucine, 2-deoxy-D-glucose or choline chloride, with or without drug, was added. At the end of a 15- or 30-min incubation at 37 °C, the vials were chilled to 2 °C and washed three times, and 1 ml of 10% Cl_3CCOOH was added. The radioactivity in an aliquot from the acid-soluble and -insoluble material was determined as described below.

Analysis of Acid-Soluble Nucleotide Pools. Uridine, adenosine, and thymidine nucleotides in the acid-soluble pools were analyzed by a modification of the method described by Greene and Magasanik (1967). HeLa cells, S_3, growing in spinner culture, were incubated at a concentration of 2×10^6 cells/ml in the presence or absence of drug for 10 min at 37 °C. [^{3}H]Uridine (15 μCi/ml), [^{3}H]adenosine (15 μCi/ml), or [^{3}H]thymidine (30 μCi/ml) was then added to the cell suspension. After 15 min, the labeling was terminated by the addition of cold Earle's solution and the cells were washed three times with the same solution. The subsequent steps were all carried out at 2 °C. The cells were lysed with 1.5 volumes of H_2O, an equal volume of 7% perchloric acid was added, the suspension was centrifuged, and the supernatant was decanted. Activated carbon (30 mg) was added to the supernatant, which was centrifuged for 10 min at 12 000*g*. The supernatant was discarded, the pellet was washed once with 0.001 N HCl, and the nucleotides were eluted from the carbon by adding 2 ml of eluant (1 N NaOH–95% C_2H_5OH–H_2O, 10:66:133, pH 11.3). After a second extraction with 1 ml of eluant, the two supernatants were combined and evaporated to dryness. The residue was dissolved in 0.1 ml of H_2O and 0.01–0.02 ml aliquots were placed on TLC plastic plates (Bakerflex cellulose PEI) and developed for 1 h in 4 M LiCl–acetic acid (1:4). The plates were air dried and cut into 1 × 2 cm rectangles, and the radioactivity was determined as described below.

Determination of Radioactivity. Radioactivity was determined in a Packard Tri-Carb liquid scintillation counter. In determining radioactivity on glass filters and in Cl_3CCOOH-soluble material, 10 ml of a cocktail composed of 7.1 mg of PPO, 6.7 ml of toluene, and 3.2 ml of Triton X-100 was used. For the determination of radioactivity in NCS-solubilized material, Aquasol (8 ml) was added after the NCS solution was neutralized with 1.5 ml of glacial acetic acid. Sections of thin plastic chromatograms were counted in 8 ml of Aquasol.

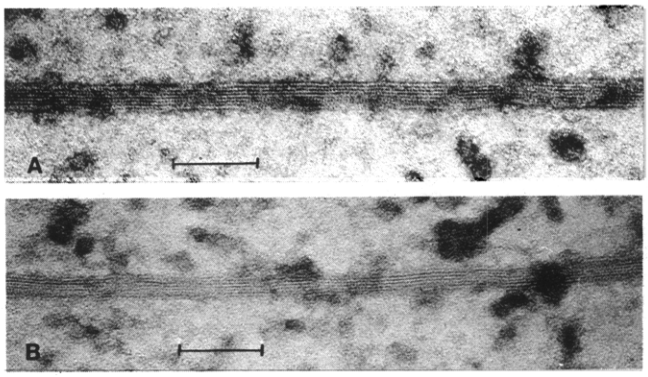

FIGURE 3: Electron microscopy of assembled microtubules (110 000×). (A) control; (B) 100 μM VP-16-213. Bar represents 0.1 μm.

Results

Effect of Podophyllotoxin and VP-16-213 on Microtubule Assembly in Vitro. Microtubule assembly in vitro was followed by monitoring the changes in turbidity that take place when unassembled tubulin polymerizes to form microtubules. Under our experimental conditions, the assembly of chicken brain tubulin is complete within 25 min (Figure 2) and polymerized tubulin can be observed by electron microscopy (Figure 3A). In the presence of 5 μM podophyllotoxin, tubulin remained in the unassembled state as determined by turbidity measurements and electron microscopy. 100 μM VP-16-213 did not affect tubulin assembly and normal polymerized microtubules were observed by electron microscopy (Figure 3B). At 10 μM, 4′-demethylepipodophyllotoxin (Figure 1), the nonglucoside congener of VP-16-213, inhibited microtubule assembly.

Effects of Podophyllotoxin and VP-16-213 on the Incorporation of Thymidine, Uridine, and Leucine into Acid-Insoluble Material. The initial rates of incorporation of labeled thymidine, uridine, and leucine into acid-insoluble material in spinner cultures of HeLa cells were used to estimate the rates of DNA, RNA, and protein synthesis, respectively. The rates of thymidine and uridine incorporation into acid-insoluble material were rapidly inhibited in cells incubated with podophyllotoxin (Figure 4A). The rate of leucine incorporation into acid-insoluble material was inhibited by 20% at 10 min and remained constant for 180 min.

In cells treated with 100 μM VP-16-213 (Figure 4B), the rate of uridine incorporation into acid-insoluble material was inhibited by 75% after a 6-min incubation. Inhibition remained at this level for 180 min. The rate of thymidine incorporation into acid-insoluble material was inhibited 65 and 97% after 6 and 180 min, respectively. After 180 min, a decrease in the rate of leucine incorporation to 50% of control cells was observed in VP-16-213 treated cells. These results suggested that VP-16-213 and podophyllotoxin acted either by inhibiting (1) uptake of thymidine and uridine into the cell, (2) conversion of thymidine and uridine to their nucleoside triphosphates, or (3) synthesis of DNA and RNA at the template or polymerase level.

Effects of Podophyllotoxin and VP-16-213 on Thymidine

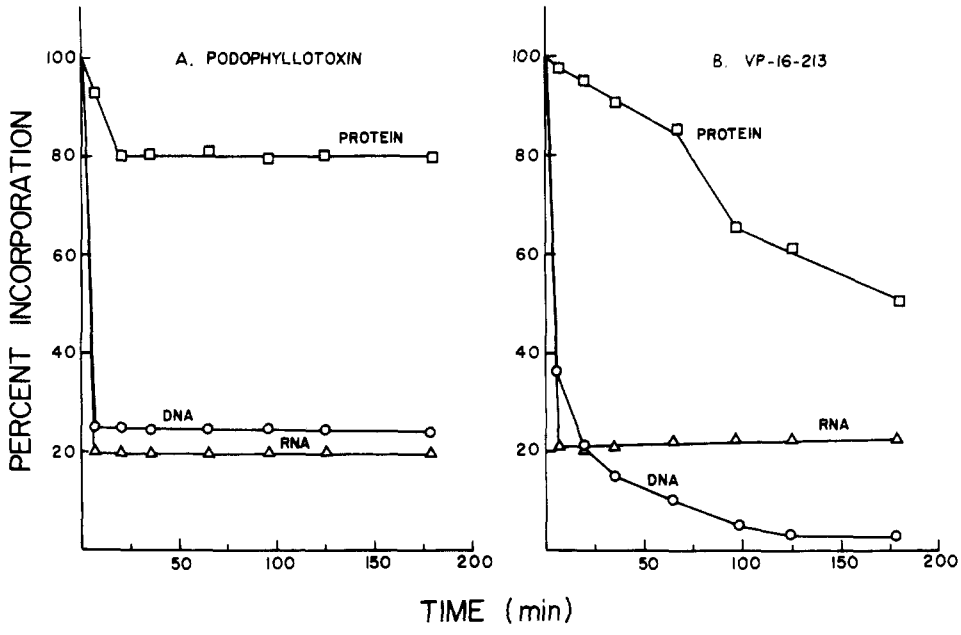

FIGURE 4: Effects of podophyllotoxin (A) and VP-16-213 (B) on precursor incorporation into acid-insoluble material. HeLa cells S_3 (4×10^5 cells/ml) were incubated at 37 °C in the presence or absence of 100 μM VP-16-213 or 100 μM podophyllotoxin. At specific time intervals, 1-ml aliquots were removed from each of three cultures and pulsed for 10 min with the appropriate isotope. The rate of incorporation of radioactivity into Cl_3CCOOH-insoluble material during each time interval is represented as the midpoint of the pulse. The percent incorporation is expressed relative to controls in which incorporation of [^{14}C]leucine (□), [^{14}C]uridine (△), or [^{14}C]thymidine (○) was 990, 1000, and 1191 cpm/ml, respectively, during the initial 10-min pulse and 1350, 998, and 1394 cpm/ml, respectively, during the final pulse.

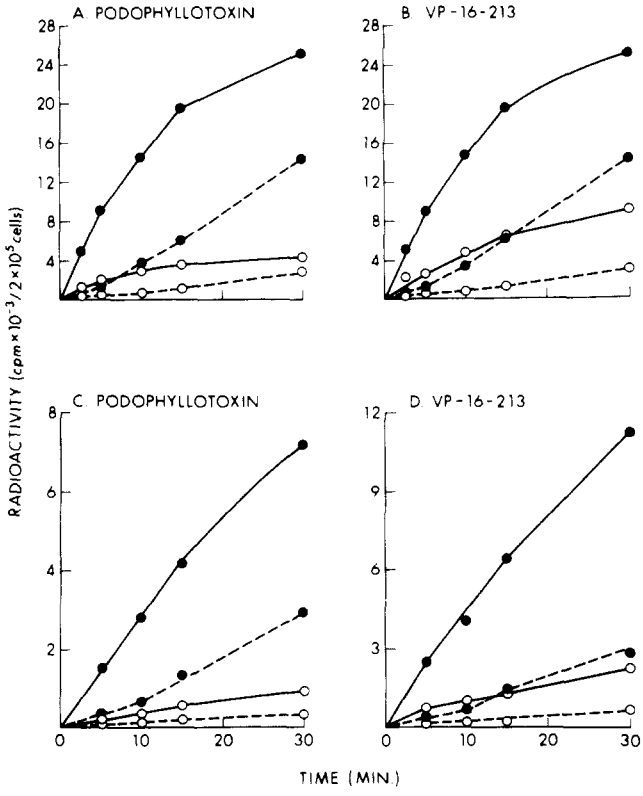

FIGURE 5: Time course for thymidine (A, B) and uridine (C, D) incorporation into Cl_3CCOOH-soluble and -insoluble material in the presence or absence of podophyllotoxin or VP-16-213. HeLa cells grown in monolayer culture were prepared as described under Materials and Methods. Cells were incubated in serum-free medium for 1 h prior to the addition of 1 μCi of [^{3}H]thymidine (A, B) or 0.2 μCi of [^{14}C]uridine (C, D) and the appropriate drug. At the end of each pulse (2.5, 5, 10, 15, or 30 min), the cells were analyzed for radioactivity in the Cl_3CCOOH-soluble (—) and -insoluble (- - -) fractions, as described under Materials and Methods. Control (●); 100 μM drug (○).

and Uridine Uptake. As an indication of exogenous thymidine uptake, the incorporation of labeled thymidine into the acid-soluble fraction of monolayer cultures of HeLa cells was followed. Within 5 min after incubation of 100 μM podophyllotoxin (Figure 5A) or 100 μM VP-16-213 (Figure 5B) with the cells, significant inhibition of incorporation of thymidine into the acid-soluble fraction, as well as the acid-insoluble fraction, of the cell was observed, indicating that these drugs inhibit thymidine uptake. The effects of podophyllotoxin and VP-16-213 on uridine uptake into the acid-soluble and -insoluble fractions are shown in Figure 5C,D. The decrease in thymidine and uridine uptake into the acid-soluble fraction of cells incubated with VP-16-213 or podophyllotoxin could account for the inhibition of thymidine and uridine incorporation into acid-insoluble material. VP-16-213 and podophyllotoxin also inhibited uridine and thymidine uptake in HeLa cells grown in spinner culture (unpublished data).

Thymidine uptake by HeLa cell monolayers was monitored as a function of exogenous thymidine concentration. In control cells, two components of uptake were observed, one of which was saturable and represents mediated uptake (Paterson et al., 1975) and the other of which was linear and directly proportional to exogenous thymidine concentration. This latter component has been described as simple diffusion by Paterson et al. (1975). In HeLa cells treated with either 100 μM podophyllotoxin (Figure 6A) or 100 μM VP-16-213 (Figure 6B), a significant decrease in the saturable rate component was observed. No change was seen in the diffusional rate component in drug-treated cells. Similar results were observed when uridine uptake studies were done (Figure 6C,D). These results demonstrate that podophyllotoxin and VP-16-213 inhibit the uptake of thymidine and uridine into HeLa cells by inhibiting the facilitated diffusional component of uptake.

Characteristics of Podophyllotoxin and VP-16-213 Inhibition of Nucleoside Uptake. The inhibition of thymidine (Figure 7) and uridine uptake into acid-soluble and -insoluble

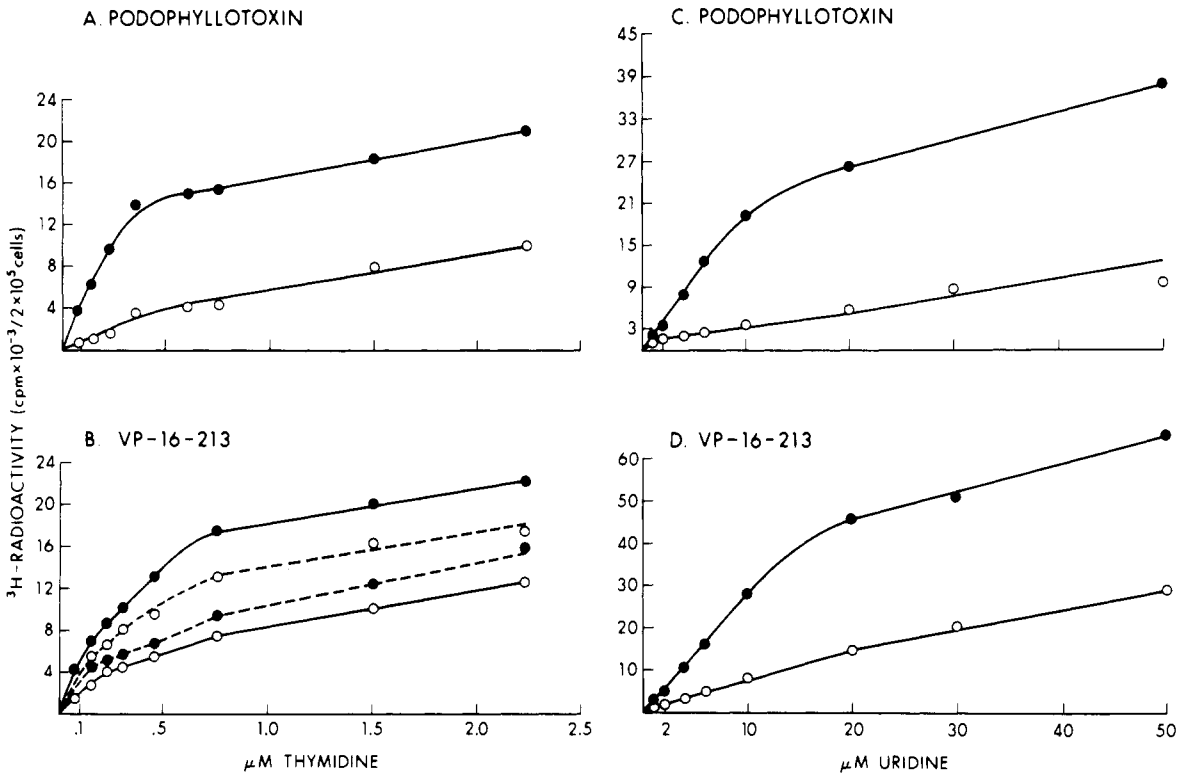

FIGURE 6: Inhibition of thymidine (A, B) or uridine (C, D) uptake by podophyllotoxin or VP-16-213 as a function of nucleoside concentration. HeLa cells grown in monolayer cultures were prepared as described under Materials and Methods. Cells were incubated in serum-free medium for 60 min prior to being pulsed for 10 min with various concentrations of [^{3}H]nucleoside (at a constant specific activity) in the presence or absence of drug. ^{3}H radioactivity was analyzed in the Cl_3CCOOH-soluble and -insoluble material as described under Materials and Methods and values are expressed as total counts per vial (2 × 10^5 cells). Control (●–●); 20 μM drug (○- - -○); 50 μM drug (●- - -●); 100 μM drug (○–○).

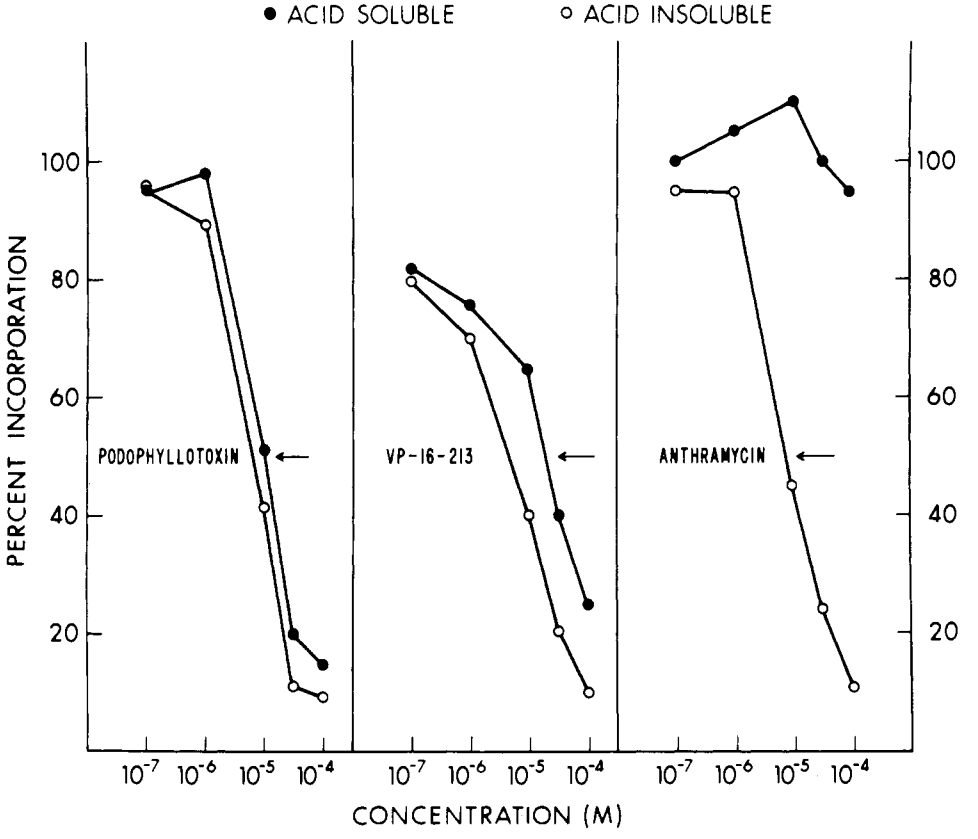

FIGURE 7: Dose-dependent inhibition of thymidine uptake by podophyllotoxin, VP-16-213, or anthramycin. HeLa cells were grown in monolayer cultures and incubated in serum-free medium in the presence or absence of drug. After 60 min, cells were pulsed for 15 min with 1 μCi/ml of [^{3}H]thymidine in the presence or absence of the drug. The cells were analyzed for ^{3}H radioactivity in the Cl_3CCOOH-soluble (●), and -insoluble (○) fractions. The arrow designates 50% inhibition.

TABLE I: Effect of Podophyllotoxin and VP-16-213 on the Uptake of Nucleosides into HeLa cells.[a]

Nucleoside	Drug Concentration (μM)	
	Podophyllotoxin	VP-16-213
Thymidine	10	25
Uridine	5	20
Adenosine	8	30
Guanosine	9	20

[a] HeLa cells grown in monolayer culture were incubated for 15 min at 37 °C with 1 μCi/ml of [^{3}H]thymidine, 0.2 μCi of [^{14}C]uridine, 1.5 μCi/ml of [^{3}H]adenosine or 1 μCi/ml of [^{3}H]guanosine in the presence or absence of drug. The experimental procedures are described under Materials and Methods. Results are expressed as the concentration of drug required for 50% inhibition of total uptake of nucleosides into cells.

TABLE II: Effect of Podophyllotoxin and VP-16-213 on the Uptake of Leucine, Choline, and 2-Deoxy-D-Glucose.[a]

Drug	Concn (μM)	% of Control		
		Leucine	Choline	2-Deoxy-D-glucose
Podophyllotoxin	100	106	107	66
	10			93
VP-16-213	100	108	113	100

[a] HeLa cells grown in monolayer culture were incubated for 30 min (15 min in leucine uptake studies) at 37 °C with 0.25 μCi of 2-deoxy-D-[^{14}C]glucose, 1 μCi of [^{3}H]choline, or 0.5 μCi of [^{14}C]leucine in the presence or absence of drug. The details of the procedures are described under Materials and Methods. Results are expressed as percent incorporation of total counts in the cell relative to controls in which incorporation of 2-deoxy-D-[^{14}C]glucose, [^{3}H]choline, and [^{14}C]leucine was 5800, 7000, and 4000 cpm/ml, respectively. In choline and 2-deoxy-D-glucose studies, greater than 90% of the radioactivity was found in the acid-soluble fraction. In leucine uptake studies, over 90% of the radioactivity in the cell was found in the acid-insoluble fraction.

material is concentration dependent. Podophyllotoxin inhibited thymidine uptake into acid-soluble and -insoluble cell fractions by 50% at 10 and 8 μM, respectively. VP-16-213 inhibited thymidine uptake into acid-soluble and -insoluble cell fractions by 50% at approximately 7 and 30 μM, respectively. Podophyllotoxin and VP-16-213 also inhibited uridine uptake into acid-soluble and -insoluble cell fractions: 50% inhibition was observed at 5 μM podophyllotoxin and 20 μM VP-16-213 (data not shown). Anthramycin (Horwitz, 1971), an established inhibitor of DNA and RNA synthesis that acts by binding to the DNA template, had no effect on thymidine and uridine uptake into the acid-soluble fraction over a wide range of drug concentrations (0.1–100 μM) but demonstrated a dose-dependent response for the inhibition of thymidine and uridine incorporation into acid-insoluble material.

The reversibility of the effects of VP-16-213 and podophyllotoxin on nucleoside uptake in HeLa cell monolayers was studied. Cells incubated for 30 min in the presence or absence of 100 μM VP-16-213 or 100 μM podophyllotoxin were washed three times with Mem. The cells were then pulsed for 15 min with either labeled thymidine or uridine. The rate of thymidine and uridine uptake in the washed cells was the same as in control cells, demonstrating that the effects of these drugs on thymidine and uridine uptake are reversible (data not shown).

As seen in Table I, podophyllotoxin and VP-16-213 inhibited the uptake of adenosine, thymidine, uridine, and guanosine. However, the uptake of leucine and choline into HeLa cells grown in monolayers was not significantly inhibited by 100 μM podophyllotoxin or VP-16-213 (Table II). Although 100 μM podophyllotoxin did inhibit the uptake of 2-deoxy-D-glucose by 34%, 10 μM podophyllotoxin and 100 μM VP-16-213 had no effect.

Effects of Podophyllotoxin and VP-16-213 on Intracellular Phosphorylation of Nucleosides. The effect of podophyllotoxin and VP-16-213 on the cellular processing of nucleosides was examined by monitoring the levels of nucleotides in acid-soluble extracts prepared from spinner cultures of HeLa cells. Acid-soluble extracts were analyzed for uridine nucleotides by thin-layer chromatography after pulsing the cells with [^{3}H]uridine for 15 min (Table III). In the untreated (control) cells, 67% of the labeled nucleotides in the acid-soluble extract was UTP, 15% UDP, and 27% a mixture of UMP and UDP-sugars. The latter two were not resolved in our chromatography system. Acid-soluble extracts, prepared from cells treated with either 100 μM podophyllotoxin or 100 μM VP-16-213, contained only 20% of the labeled nucleotides present in the control cells but the ratio of UTP, UDP, and UMP plus UDP-sugars to total nucleotides was quite similar to that of control cells. This suggests that these drugs have an insignificant effect on uridine kinase(s), since drug-treated cells were still able to phosphorylate uridine to the di- and triphosphate nucleotides.

Acid-soluble extracts prepared from cells pulsed with [^{3}H]thymidine for 15 min were analyzed for thymidine nucleotides. In the acid-soluble extract of the control cells, 89% of the thymidine-containing nucleotides was in the form of TTP, 6% in the form of TDP and 4% in the form of TMP (Table III). Cells treated with 100 μM podophyllotoxin or 100 μM VP-16-213 for 15 min showed a significant decrease in the total concentration of thymidine nucleotides; yet, only a slight change was observed in the ratio of TTP, TDP, and TMP present in the acid-soluble extracts as compared to control cells. Similar results to those observed with thymidine and uridine were seen with adenosine nucleotides (data not shown). Cells treated with anthramycin demonstrated no change in either the total intracellular thymidine, uridine, or adenosine nucleotide levels or in the ratios of mono-, di-, and triphosphates, as compared to the control cells. The results with anthramycin are consistent with the fact that anthramycin does not inhibit thymidine or uridine uptake.

At 2 °C, cells continue to take up and phosphorylate exogenous uridine but they do not synthesize RNA at this temperature (Scholtissek, 1967; Kunimoto et al., 1974). When cells were pulsed with [^{3}H]uridine at 2 °C in the presence or absence of drug, there was a decrease in the total concentration of intracellular nucleotides as compared to incubation at 37 °C. However, the ratio of mono-, di-, and triphosphates in untreated and drug-treated cells incubated at 2 °C was identical (data not shown). This is the same result observed after incubation at 37 °C, a temperature at which both uridine uptake and RNA synthesis take place.

Effects of Podophyllotoxin and VP-16-213 on RNA Synthesis. Although podophyllotoxin and VP-16-213 inhibit uridine uptake, one cannot exclude the possibility that these drugs may also affect RNA synthesis. To investigate this possibility, the effects of podophyllotoxin and VP-16-213 on

TABLE III: Effect of Podophyllotoxin and VP-16-213 on the Concentration of Intracellular Nucleotides.[a]

Nucleotides	No Addition	VP-16-213		Podophyllotoxin		Anthramycin
		100 μM	10 μM	100 μM	10 μM	50 μM
UTP	1762 (67)	261 (54)	887 (65)	215 (51)	615 (65)	1733 (72)
UDP	134 (5)	44 (9)	87 (6)	29 (7)	46 (4)	128 (5)
UMP and UDP-sugars	703 (27)	177 (36)	373 (28)	171 (41)	282 (29)	522 (22)
TTP	6892 (89)	4071 (88)	5253 (89)	4124 (82)	5765 (92)	6917 (92)
TDP	474 (6)	246 (5)	249 (4)	676 (13)	239 (4)	335 (4)
TMP	347 (4)	387 (6)	351 (6)	214 (4)	250 (4)	266 (4)

[a] HeLa cells S_3, (2×10^6 cells/ml) were incubated in the presence or absence of drug at 37 °C. After 10 min, either [^{3}H]thymidine or [^{3}H]uridine was added for 15 min and the acid-soluble nucleotide pools were analyzed as described under Materials and Methods. Values are presented as counts per minute and the numbers in parentheses represent the percentage of total radioactivity in each nucleotide.

the incorporation of uridine nucleotides from their intracellular pools into acid-insoluble RNA was followed. Spinner cultures of HeLa cells were incubated with [^{3}H]uridine for 30 min at 2 °C. After incubation in the cold, the cells were washed in the cold and incubated at 37 °C in fresh medium in the presence or absence of drug but without additional uridine. As described previously, at 2 °C the cells phosphorylate uridine into its respective nucleotides, but RNA synthesis is inhibited. When the cells are shifted from 2 to 37 °C, RNA synthesis resumes. The radioactivity present in whole cells and incorporated into acid-insoluble material was monitored at different time intervals after the shift to 37 °C.

As seen in Figure 8, total radioactivity in the cells is at a constant level during the 60-min incubation at 37 °C in the presence or absence of 100 μM podophyllotoxin, 100 μM VP-16-213, or 50 μM anthramycin. There is also no significant difference in the incorporation of labeled uridine nucleotides into acid-insoluble material between control cells and cells treated with podophyllotoxin or VP-16-213. In contrast, cells incubated with 50 μM anthramycin incorporated 25% of their radioactivity into acid-insoluble material as compared to control cells. These results are consistent with the fact that anthramycin inhibits RNA synthesis at the template level and indicate that podophyllotoxin and VP-16-213 do not inhibit RNA synthesis within 30 min.

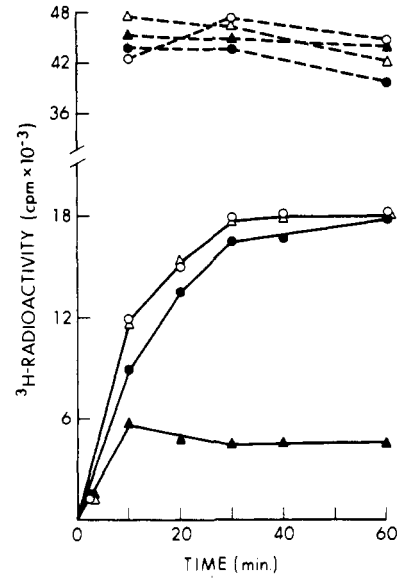

FIGURE 8: Effect of podophyllotoxin and VP-16-213 on RNA synthesis in HeLa cells. HeLa cells S_3 (2×10^6 cells/ml) were incubated with [^{3}H]uridine (1.5 μCi/ml) at 2 °C for 30 min, washed twice with cold Mem, and divided into four aliquots: (1) Control cells (○); (2) 100 μM VP-16-213 (△); (3) 100 μM podophyllotoxin (●); (4) 50 μM anthramycin (▲). During incubation at 37 °C, 2-ml aliquots were removed at the specified time intervals and analyzed for radioactivity in whole cells (- - -) and in Cl_3CCOOH-insoluble fractions (—).

Discussion

Although mammalian cells do not require exogenous nucleosides for growth, cells are able to transport these nucleosides across their membrane, to phosphorylate and to utilize them in nucleic acid synthesis. Inhibitors of nucleoside transport can be divided into two subclasses, compounds like nitrobenzylthioguanosine and nitrobenzylthioinosine (Paterson et al., 1975), which are chemically related to nucleosides, and those which are chemically unrelated to nucleosides. The latter class includes colchicine and podophyllotoxin (Mizel and Wilson, 1972), aflatoxin (Kunimoto et al., 1974), cytochalasin B and persantin (Plagemann and Richey, 1974), colcemid (Plagemann and Erbe, 1974), and phloretin (LeFevre, 1954). Several solvents, such as dimethyl sulfoxide (Collins and Roberts, 1971), ethanol, and phenethyl alcohol (Plagemann and Erbe, 1974), inhibit transport of a large variety of cellular substrates, including nucleosides. However, the inhibition of biological transport by these solvents requires very high concentrations (0.01–1 M) and is related to a nonspecific lipophilic interaction with the membrane.

Our results demonstrate that VP-16-213 and podophyllotoxin, which are chemically unrelated to nucleosides, reversibly inhibit the uptake of thymidine, uridine, adenosine, and guanosine, which have distinct transport pathways (Mizel and Wilson, 1972; Paterson et al., 1975) in HeLa cells. At 100 μM, VP-16-213 does not inhibit 2-deoxy-D-glucose, choline, or leucine uptake into HeLa cells. At 100 μM, podophyllotoxin also does not inhibit choline or leucine uptake but demonstrates a partial inhibition (34%) of 2-deoxy-D-glucose uptake in HeLa cells. These results suggest that at 100 μM, VP-16-213 and podophyllotoxin are selective inhibitors of nucleoside transport.

Current evidence suggests that nucleoside transport consists of two phases (Plagemann and Erbe, 1972; Paterson et al., 1975). In the presence of low concentrations of exogenous nucleosides, HeLa cells transport nucleosides into the cell by facilitated diffusion, a saturable carrier-mediated process. At higher concentrations, thymidine and uridine are transported by simple diffusion. Our results indicate that VP-16-213 and podophyllotoxin inhibit the saturable component (i.e., facilitated diffusion) without affecting the linear component (i.e., simple diffusion).

If these agents inhibit phosphorylation of nucleosides, one

would expect the linear component, as well as the saturable component of uptake, to be affected. Furthermore, our data indicate that, in the presence of podophyllotoxin and VP-16-213, cells are able to phosphorylate nucleosides to their respective nucleotides. The ratio of tri-, di-, and monophosphates is similar in control and drug-treated cells. These results, coupled with current evidence (Plagemann and Richey, 1974) that phosphorylation is essential for nucleoside trapping inside the cell but not essential for nucleoside transport, suggest that podophyllotoxin and VP-16-213 inhibit transport without significantly inhibiting phosphorylation.

Although there is no formal analogy established between transport processes and enzyme kinetics, several investigators have calculated a V_{max} and K_M for nucleoside uptake in HeLa cells (Paterson et al., 1975; Mizel and Wilson, 1972; Kunimoto et al., 1974). In these studies, the K_M constant is represented as the permeant concentration at which uptake rate is half-maximal. In our studies, the K_M for nucleoside transport was calculated by subtracting the simple diffusional component from the total uptake, thereby determining the K_M for the saturable component of transport. Values of 0.5 and 12 μM were calculated for thymidine and uridine transport, respectively, and these values are consistent with published reports (Paterson et al., 1975; Mizel and Wilson, 1972).

We have examined the effects of VP-16-213 and podophyllotoxin on microtubule assembly in vitro in order to determine if the cytotoxic effects seen with VP-16-213 are due to an inhibition of microtubule assembly. In contrast to podophyllotoxin which completely inhibits the in vitro assembly of microtubules at 5 μM, VP-16-213, at 100 μM, has no effect. Furthermore, 4′-demethylepipodophyllotoxin, the nonglucoside congener of VP-16-213, inhibits nucleoside transport in HeLa cells but unlike VP-16-213, inhibits microtubule assembly in vitro. These results suggest that the glucoside moiety is responsible for the inactivity of VP-16-213 as an inhibitor of microtubule assembly and, unless the glucoside moiety of VP-16-213 is cleaved by cellular enzymes, the cytotoxicity of this drug cannot be due to its effect on microtubules. Recent studies (Allen et al., 1976) have identified 4′-demethylepipodophyllic acid glucoside as the major urinary metabolite of VP-16-213 in man, thereby suggesting that the glucoside moiety is not cleaved intracellularly.

Several membrane properties, such as nucleoside transport in HeLa cells and receptor mobility in lymphocytes, are influenced by agents which inhibit microtubule assembly. The effects of agents like podophyllotoxin or colchicine on nucleoside transport may be related to their action on microtubules or may be a result of a direct interaction of these agents with transport systems. Our results suggest that the effects of podophyllotoxin on nucleoside transport are not associated with its effects on microtubule assembly, since VP-16-213 inhibits nucleoside transport but has no effect on microtubule assembly in vitro. These results relate to the report by Mizel and Wilson (1972) that the effects of colchicine on microtubules are not associated with the inhibition of nucleoside transport by the drug.

In conclusion, although both podophyllotoxin and VP-16-213 inhibit nucleoside transport, these drugs exhibit other biological properties which are quite distinct. Podophyllotoxin inhibits microtubule assembly in vitro, while VP-16-213 has no effect on microtubule assembly. In the following paper of this issue (Loike and Horwitz, 1976), we report that VP-16-213 induces single-stranded breaks in DNA in HeLa cells, an effect which may explain the cytotoxicity of VP-16-213. In contrast to VP-16-213-treated cells, cells treated with podophyllotoxin maintain DNA integrity and show no evidence of single stranded breaks. Furthermore, VP-16-213 exerts a greater inhibition on the incorporation of thymidine into acid-insoluble material than podophyllotoxin (Figure 4); yet, VP-16-213 is a less potent inhibitor of thymidine transport (Table I). These results support the idea that VP-16-213 exerts a DNA effect not seen in podophyllotoxin-treated cells. The differences in biological activity between VP-16-213 and podophyllotoxin introduce an interesting group of drugs for studying the relationship between chemical modifications and biological properties.

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References

Allen, L. M., Marcks, C., and Creaven, P. J. (1976), *Proc. Am. Assn. Cancer Res. 17,* 6 (Abstract).

Collins, F. D., and Roberts, W. K. (1971), *Biochim. and Biophys. Acta 233,* 459.

Creaven, P. J., Allen, L. M., and Alford, D. A. (1975), *Clin. Pharmacol. Ther. 17,* 232.

Dombernowsky, P., Nissen, N. I., and Larsen, V. (1972), *Cancer Chemother. Rep., Part 1 56,* 71.

Feit, H., Dutton, G. R., Barondes, S. H., and Shelanski, M. L. (1971), *J. Cell Biol. 51,* 138.

Forsdyke, D. R. (1971), *Biochem. J. 125,* 721.

Gaskin, F., Cantor, C. R., and Shelanski, M. L. (1974), *J. Mol. Biol. 89,* 737.

Greene, R., and Magasanik, B (1967), *Mol. Pharmacol. 3,* 453.

Grieder, A., Maurer, R., and Stähelin, H. (1974), *Cancer Res. 34,* 1788.

Hartwell, J. L., and Schrecker, A. W. (1958), *Fortschr. Chem. Org. Naturst. 15,* 83.

Horwitz, S. B. (1971), *Prog. Mol. Subcell. Biol. 2,* 40.

Horwitz, S. B., Chang, C., and Grollman, A. P. (1971), *Mol. Pharmacol. 7,* 632.

Huang, C. C., Hou, Y., and Wang, J. J. (1973), *Cancer Res. 33,* 3123.

Kelly, M. G., and Hartwell, J. L. (1954), *J. Natl. Cancer Inst. 14,* 967.

Klevecz, R. R., and Stubblefield, E. (1967), *J. Exp. Zool. 165,* 259.

Kunimoto, T., Kurimoto, Y., Aibara, K., and Miyaki, K. (1974), *Cancer Res. 34,* 968.

LeFevre, P. G. (1954), *Symp. Exp. Biol. 8,* 118.

Loike, J. D., and Horwitz, S. B. (1976), *Biochemistry 15,* (following paper in this issue).

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.

Mizel, S. B., and Wilson, L. (1972), *Biochemistry 11,* 2573.

Nissen, N. I., Larsen, V., Pedersen, H., and Thomsen, K. (1972), *Cancer Chemotherap. Rep. Part 1 56,* 769.

Paterson, A. R. P., Kim, S. C., Bernard, O., and Cass, C. E. (1975), *Ann. N.Y. Acad. Sci. 255,* 402.

Plagemann, P. G. W., and Erbe, J. (1972), *J. Cell Biol. 55,* 161.

Plagemann, P. G. W., and Erbe, J. (1974), *Cell 2,* 71.

Plagemann, P. G. W., and Richey, D. P. (1974), *Biochim. Biophys. Acta 344,* 263.

Plagemann, P. G. W., and Roth, M. F. (1969), *Biochemistry*

8, 4782.
Scholtissek, C. (1967), *Biochim. Biophys. Acta 145*, 228.
Shelanski, M. L., Gaskin, F., and Cantor, C. R. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70*, 765.
Stähelin, H. (1973), *Eur. J. Cancer 9*, 215.
Wilson, L., Bamburg, J. R., Mizel, S. B., Grisham, L. M., and Creswell, K. M. (1974), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 33*, 158.

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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