Comparison of Cytotoxicity and DNA Breakage Activity of Congeners of Podophyllotoxin Including VP16-213 and VM26: A Quantitative Structure–Activity Relationship†

Byron H. Long,* Steven T. Musial, and Michael G. Brattain

ABSTRACT: Fourteen congeners of podophyllotoxin were evaluated for their abilities to induce DNA breakage and inhibit growth of A549 human lung adenocarcinoma cells. Among the congeners studied were VP16-213, VM26, α-peltatin, β-peltatin, and picropodophyllotoxin. Alkaline elution methods were used to assess DNA break frequencies following 1-h exposure to different concentrations of the congeners. DNA breakage was dependent upon drug concentration and was detectable when cells were exposed for 1 h to concentrations of VM26 as low as 0.05 μM. DNA breaks formed rapidly in cells after addition of drug but increased little after 30 min of continuous exposure. Repair of drug-induced DNA breaks was equally rapid with repair of 90% of the breaks occurring within 1 h following removal of the drug. Relationships between the structures of the congeners and the resulting DNA breakage activities were obtained, which correlated well with the cytotoxicity. The data suggest that a free hydroxyl group at the 4′-position is essential for DNA breakage activity, epimerization at the 4-position of the podophyllotoxin rings enhances activity, glucosylation of the hydroxyl group at the 4-position diminishes activity, aldehyde condensation with the glucose moiety greatly enhances activity, and the structure of the group associated with the resulting acetal linkage influences DNA breakage activity. These studies present quantitative data supporting and expanding upon the structure–activity relationship first proposed by Loike and Horwitz [Loike, J. D., & Horwitz, S. B. (1976) *Biochemistry 15*, 5443–5448].

Podophyllotoxin is a naturally occurring antibiotic that inhibits assembly of microtubules into the mitotic apparatus by binding to the tubulin dimer (Wilson & Bryan, 1974; Wilson & Friedkin, 1967) in a manner more rapid and more reversible than colchicine (Cortesse et al., 1977). Although it was evaluated as a chemotherapeutic agent against cancer, its toxic side effects precluded further development as an anticancer drug for humans (Savel, 1964, 1966). Attempts to chemically modify podophyllotoxin to obtain a more tolerated agent have resulted in the synthesis of two derivatives of podophyllotoxin, VP-16-213 (etoposide) and VM-26 (teniposide) (Stähelin, 1970, 1972, 1973), which have demonstrated effectiveness in clinical studies with few toxic side effects [for a review, see Issell (1982)].

At first, it was assumed that these semisynthetic derivatives of podophyllotoxin also acted as antimicrotubule agents (Keller-Juslén et al., 1971), but it was soon recognized that they differed from podophyllotoxin by inhibiting cell cycle progression prior to mitosis (Stähelin, 1970, 1972). In fact, it is concluded that VM26 and VP16-213 possess mechanisms of action that block cells in the G_2 phase of the cell cycle, in contrast to podophyllotoxin (Grieder et al., 1974; Krishan et al., 1975; Misra & Roberts, 1975; Kalwinsky et al., 1983).

Evidence now indicates that the cytotoxicity produced by low concentrations of VP16-213 and VM26 may be due to DNA breakage resulting from the exposure of cells to the drugs. Loike & Horwitz (1976b) first reported that VP16-213 induces DNA breaks in vivo but not in vitro as assayed by alkaline sucrose gradient sedimentation but at doses much higher than required for cytotoxicity. VM26 has a similar effect on DNA (Roberts et al., 1980). Employing alkaline elution technology, Wozniak & Ross (1983) recently demonstrated drug-induced single-strand breakage by exposure of L1210 cells to VP16-213 for 1 h at concentrations as low as 1 μM. A study relating drug structure to in vivo DNA breakage activity was conducted by Loike & Horwitz (1976b) using sucrose gradient sedimentation methods. They found that congeners of VP16-213 require a free hydroxyl group at the 4′-position for DNA breakage activity and this activity can be influenced by the configuration at the 4-carbon position. Although these studies clearly showed a structure–activity relationship between different congeners of VP16-213, the method employed in these studies lacked the sensitivity required to quantitatively analyze effects of subtle structural changes on DNA breakage activity.

The present study was conducted to more accurately measure DNA breakage activity produced by different congeners of podophyllotoxinn in a human cancer line using alkaline elution techniques to quantitate DNA break frequencies and to determine if a correlation exists between DNA breakage and cytotoxicity. Our findings corroborate those of Loike & Horwitz (1976b) and, in addition, demonstrate that glycoside substitution has a pronounced effect upon in vivo DNA breakage activity.

Materials and Methods

Materials. Podophyllotoxin was purchased from Aldrich; 4′-demethylpodophyllotoxin, epipodophyllotoxin, 4′-demethylepipodophyllotoxin, 4′-demethylpodophyllotoxin β-D-glucoside, 4′-demethylepipodophyllotoxin β-D-glucoside, 4′-demethylpodophyllotoxin benzylidene-β-D-glucoside, and 4′-demethylepipodophyllotoxin benzylidene-β-D-glucoside were gifts from Drs. A. von Wartburg and H. Stähelin, Sandoz, Switzerland; picropodophyllotoxin, deoxypodophyllotoxin, α-peltatin, and β-peltatin were obtained from Dr. M. Suffness of the Natural Products Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD; 4′-demethylepipodophyllotoxin ethylidene-β-D-glucoside (VP16-213) and 4′-demethylepipodophyllotoxin thenylidene-β-D-glucoside (VM26) were obtained from Bristol Myers, Syra-

† From the Bristol-Baylor Laboratory, Department of Pharmacology, Baylor College of Medicine, Houston, Texas 77030. *Received August 17, 1983.* A preliminary report of this work was presented at the 67th Annual Meeting of the Federation of American Societies for Experimental Biology (Long et al., 1983).

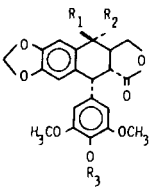
0006-2960/84/0423-1183$01.50/0

cuse, NY. [*methyl*-^{3}H]Thymidine (2 Ci/mmol) and 2-[^{14}C]thymidine (60 mCi/mmol) were purchased from Schwarz/Mann, liquiscint scintillation fluid was from National Diagnostics, polycarbonate filters (2.0 μm, 25-mm diameter) were obtained from Nucleopore, and tetrapropylammonium hydroxide was a product of R.S.A. Corp.

Cell Cultures and Radioactive Labeling. Human lung adenocarcinoma cell line A549 was maintained in Dulbecco's MEM (4.5 g/L glucose) (Gibco) containing 10% fetal bovine serum (Gibco) in the presence of penicillin and Kanamycin. Duplicate cultures intended for alkaline elution were started 3 days prior to drug treatment and incubated separately with 0.01 μCi/mL [^{14}C]thymidine and 0.1 μCi/mL [^{3}H]thymidine for 48 h prior to harvesting for drug treatment. The flasks were half-confluent with cells in exponential growth when the cells were released from flasks with trypsin–EDTA (Gibco).

Alkaline Elution Assay for Single-Strand DNA Breaks. ^{14}C-Labeled cells suspended at 5 × 10^5 cells/mL in fresh medium containing serum were either irradiated at 0 °C with various doses of γ-radiation from a ^{60}Co source in a Gammacell 220 irradiator (Atomic Energy of Canada) to introduce random single-strand DNA breaks for the preparation of a radiation/breakage standard curve or were incubated for 1 h at 37 °C with various dilutions of drugs as specified under Results. The drugs were dissolved as 10 mM stock solutions in Me_2SO for storage at −10 °C for no longer than 3 weeks. Intermediate dilutions were made in sterile water immediately before addition to cell suspensions. Incubations with drugs were terminated by placing tubes of cells on ice, centrifuging, and resuspending in fresh cold serum. Drug-treated or irradiated cells containing [^{14}C]DNA were layered over polycarbonate filters in 25-mm Swinnex filter holders affixed to 30-mL syringe barrels as described by Kohn et al. (1981) and washed twice by gravity filtration at 4 °C with 10 mL of cold phosphate buffered saline (PBS) (0.15 M NaCl, 0.01 M NaH_2PO_4, pH 7.5). Cells containing [^{3}H]DNA were irradiated on ice with 300 rd of γ-radiation and distributed among the filters in aliquots containing 5 × 10^5 cells to provide an internal standard for comparison purposes (Ewig & Kohn, 1978; Ross et al., 1978; Kohn et al., 1979). The cells were again washed with PBS and lysed on the filters at room temperature by addition of 3 mL of SDS–EDTA lysis solution (2% sodium dodecyl sulfate, 25 mM EDTA, pH 9.7) followed by 2 mL of SDS–EDTA solution containing 0.5 mg/mL proteinase K (E. Merck, Darmstadt) that was heated at 56 °C for 1 h and cooled prior to use (Kohn et al., 1981). DNA was eluted from the filters with 30 mL of tetrapropylammonium hydroxide–EDTA elution buffer, pH 12.1, at a rate of 40 μL/min by using a peristaltic pump. Fractions were collected directly into scintillation vials every 90 min. Filters, tubing, lysis fractions, and eluted fractions were processed for radioactive counting as previously described (Kohn et al., 1974). Resulting ^{14}C and ^{3}H cpm of the eluted fractions were expressed as percents of the total cpm resulting from the sum of cpm remaining on the filter and tubing, present in the lysis flow through, and collected in the eluted fractions for each sample. Elutions of [^{14}C]DNA from drug-treated cells were compared with elutions of [^{14}C]DNA from cells exposed to various doses of ionizing radiation to obtain rad equivalents (Kohn et al., 1981) and DNA single-strand break frequencies as described by Long & Brattain (1984).

Cytotoxicity. Cytotoxicities of the different congeners were assessed by comparing cell growth rates with those of controls after a 1-h incubation with drug. Cells at concentrations of 1 × 10^5 cells/mL in 5-mL aliquots were added to 25-cm^2 flasks



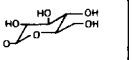
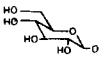
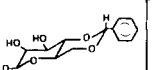
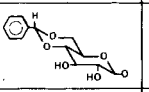
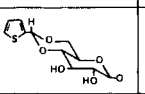
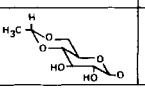
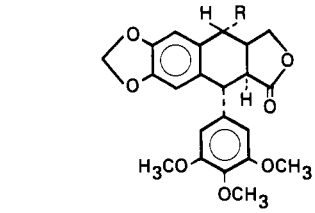
Congener	Abbreviation	R_1	R_2	R_3
deoxypodophyllotoxin	DOP	H	H	CH_3
podophyllotoxin	P	H	OH	CH_3
epipodophyllotoxin	EP	OH	H	CH_3
4' - demethylpodophyllotoxin	DMP	H	OH	H
4' - demethylepipodophyllotoxin	DMEP	OH	H	H
4' - demethylpodophyllotoxin glucoside	DMPG	H		H
4' - demethylepipodophyllotoxin glucoside	DMEPG		H	H
4' - demethylpodophyllotoxin benzylidine glucoside	DMPBG	H		H
4' - demethylepipodophyllotoxin benzylidine glucoside	DMEPBG		H	H
4' - demethylepipodophyllotoxin thionylidine glucoside	VM		H	H
4' - demythylepipodophyllotoxin ethylidine glucoside	VP		H	H

FIGURE 1: Congeners with the same multiple ring structure as podophyllotoxin but altered composition or steric orientation of peripheral groups.

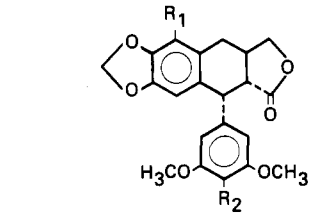
on day 1. On day 2 the medium in each flask was replaced with medium containing various dilutions of drug. Following a 1-h incubation at 37 °C the drug-containing medium was replaced with fresh medium and the incubation continued without drug for 5 days. Surviving cells were suspended with trypsin–EDTA and counted with a hemocytometer in the presence of trypan blue. Average counts for drug-treated cells were expressed as a percentage of the average number of counts for untreated control cells from four flasks for each experiment.

Results

Chemical Structures of the Congeners. The congeners chosen for this study either have the same multiple ring structures as podophyllotoxin but differ with respect to composition or steric orientation of peripheral groups (Figure 1) or have minor modifications of the podophyllotoxin rings including steric orientation of one of the ring carbon–carbon bonds (Figure 2, PP) and substitution on different rings (Figure 2, α- and β-PEL). Of the congeners shown in Figures 1 and 2, only DOP, P, EP, PP, and β-PEL have methoxy groups in the 4′-position. All the other congeners have a free phenolic group at this position. The other modified site is the 4-carbon position of the C ring, which is unsubstituted in the peltatins (Figure 2) and DOP (Figure 1) or substituted in both steric configurations similar and epi to podophyllotoxin. Substituted stereoisomers at this position include free secondary alcohols (PP, P, EP, DMP, and DMEP), unmodified



Congener	Abbreviation	R
Picropodophyllin	PP	OH



Congener	Abbreviation	R_1	R_2
α - peltatin	α - PEL	OH	OH
β - peltatin	β - PEL	OH	OCH_3

FIGURE 2: Congeners with minor modifications of the podophyllotoxin rings.

Table I: Antimicrotubule Activity of Podophyllotoxin Congeners

congener	inhibition of microtubule assembly (ID_{50}) (μM)[a]	tubulin binding (K_i) (μM)[b]
P	0.6[c,d]	0.51
PP	30[c]	10.1
DOP	0.5[c,d]	0.54
α-PEL	0.5[d]	
β-PEL	0.7[d]	0.12
EP	5[c,d]	12.1
DMP	0.5[d]	0.65
DMEP	2[d]	
VM26	>>100[d]	
VP16-213	>>100[c,d]	

[a] ID_{50} values were taken from the data of Brewer et al. (1979) and Loike et al. (1978) and represent the concentrations of drugs necessary to inhibit chicken brain microtubule assembly by 50% during a 30-min incubation period at 37 °C. [b] K_i values were determined by Kelleher (1977) from Linweaver-Burk plots of the competitive inhibition of [^{3}H]colchicine binding to mouse brain tubulin by the different congeners. [c] Brewer et al. (1979). [d] Loike et al. (1978).

glucosides (DMPG and DMEPG), and glucosides substituted at the 4,6-O positions by acetal linkages (DMPBG, DMEPBG, VM26, and VP16-213) (Figure 1). Kelleher (1977), Loike et al. (1978) and Brewer et al. (1979) reported that many of these congeners have antimicrotubule activity similar to the activity of the parent compound, podophyllotoxin. A comparison of antimicrotubule activities of the congeners is provided in Table I. Although no reference could be found for the antimicrotubule activity of the glycosylated congeners used in our study, Kelleher (1977) found that glycosylation of β-peltatin and podophyllotoxin greatly diminished antimicrotubule activity whereas demethylation and epimerization had little effect on this activity (Table I).

Quantitation of in Vivo DNA Breakage Produced by the Congeners of Podophyllotoxin. Alkaline elution of DNA from filters has proven to be a reliable and sensitive method for assessing DNA breakage (Kohn et al., 1981) and has been used recently to demonstrate DNA breakage produced by very low concentrations of VP16-213 (Wozniak & Ross, 1983; Kalwinsky et al., 1983). When assayed under deproteinizing

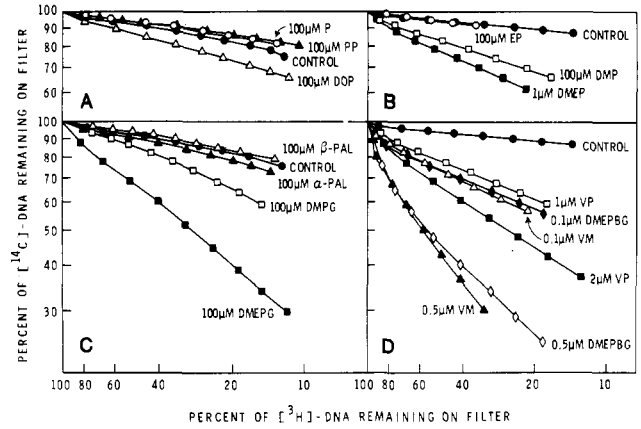


FIGURE 3: Representative alkaline elution curves of DNA from cells incubated with different congeners of podophyllotoxin. A549 cells containing [^{14}C]DNA were incubated for 1 h at 37 °C with different concentrations of the congeners, then added to polycarbonate filters along with 300-rd irradiated cells containing [^{3}H]DNA and lysed in the presence of proteinase K. Radioactivities in the eluted fractions collected at 1.5-h intervals were plotted as described under Materials and Methods. (A) P, PP, and DOP; (B) EP, DMP, and DMEP; (C) α-PEL, β-PEL, DMPG, and DMEPG; (D) DMEPBG, VM26, and VP16-213.

conditions, DNA containing random single-strand breaks eluted from polycarbonate filters at pH 12.1 to yield nearly linear curves when plotted against the elution of DNA from irradiated cells as a reference (unpublished results). Random single-strand breaks produced in DNA by exposing cells to ionizing radiation can be used as a calibration for calculating single-strand break frequency.

When the slopes of such elution curves are plotted against the corresponding radiation doses, a linear relationship is obtained (unpublished results), as previously reported by Kohn et al. (1981). This calibration curve is then used to determine rad equivalents for elution slopes resulting from drug treatment of A549 cells. Rad equivalents are converted into single-strand break production by the formula 1 rd = 9×10^{-10} single-strand break/nucleotide (Kohn et al., 1976).

Representative alkaline elution curves of DNA from cells exposed to various concentrations of the different congeners are shown in Figure 3. Panels A–C of Figure 3 display curves obtained when cells were exposed to congeners displaying little or no activity at 100 μM, the highest concentration used in this study. At this concentration the cells were simultaneously exposed to 1% Me_2SO as a solvent for stock solutions of the congeners, which has been reported to cause DNA breaks in erythroleukemia cells (Scher & Friend, 1978), but 1% Me_2SO alone had no effect on DNA integrity within cells exposed only for 1 h (unpublished results). Panel D of Figure 3 demonstrates that VP16-213, VM26, and DMEPBG are capable of causing DNA single-strand breaks at very low concentrations; in fact, breaks are detectable in cells exposed to less than 50 nM concentrations of VM26 or DMEPBG for 1 h (unpublished results). It should be noted that these elution curves are highly linear with correlation coefficients mostly above 0.999, as determined by linear regression analysis. It is only when breakage becomes extensive (e.g., 0.5 μM VM26 and DMEPBG, Figure 3D) that the elution profiles become curvilinear. This linearity seen at lower levels of breakage suggests that break formation is random throughout the genome. Although VP16-213-induced DNA–protein cross-link formation has been demonstrated (Wozniak & Ross, 1983), their interference in the assay is precluded by the employment of proteinase K in the lysis step and polycarbonate filters. No evidence for DNA–DNA cross-link formation has been found.

Table II: Single-Strand Break Frequency Expressed in Breaks per 10^8 Nucleotides[a]

congener	drug dose 10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M
P	<0.5			
PP	<0.5			
DOP	1.2 ± 1.0	<0.5		
α-PEL	1.4 ± 0.4	<0.5		
β-PEL	<0.5			
EP	<0.5			
DMP	12.0 ± 1.5	2.3 ± 0.7	<0.5	
DMEP	>30	11.5 ± 2.3	4.8 ± 1.5	<0.5
DMPG	3.2 ± 2.5	<0.5		
DMEPG	11.1 ± 3.8	<0.5		
DMPBG	>30	8.0 ± 3.1	0.9 ± 1.1	<0.5
DMEPBG		>30	23.2 ± 6.4	6.9 ± 1.9
VM26		>30	26.6 ± 2.9	5.3 ± 2.4
VP16-213		>30	5.5 ± 2.1	<0.5

[a] Break frequencies and standard deviations were determined from at least three separate experiments for each congener, where A549 cells were exposed for 1 h to drugs at the concentration shown. Break frequencies were calculated from rad equivalents, which were obtained from slopes of elution curves as described in the text. Values below 0.5 or above 30 breaks/10^8 nucleotides exceed limits for reliable evaluation of break frequencies under the conditions employed for the assay.

Therefore, the criteria for the quantification (i.e., random DNA breakage and no interference by cross-link formation) have been realized (Kohn et al., 1981).

Single-strand DNA breakage was determined from slopes of the elution curves as described under Materials and Methods. Break frequencies expressed as breaks per 10^8 nucleotides are shown in Table II. It is evident from Table II and Figure 3 that P, PP, β-PEL, and EP have no detectable DNA breakage activity at 100 μM, whereas DOP and α-PEL display slight activity at 100 μM. Demethylation of podophyllotoxin at the 4′-position (DMP) increases that activity and epimerization at the 4-carbon (DMEP) further increases breakage activity of the congener, but glycosylation of the hydroxyl group at the 4-position (DMPG and DMEPG) decreases breakage activity. However, substitution at the 4,6-O positions of the glucoside by acetal linkages formed by addition of benzaldehyde (DMPBG and DMEPBG), acetaldehyde (VP16-213), or thiophene-2-carboxaldehyde (VM26) greatly enhances breakage activity. A comparison of break frequencies resulting from exposure of cells for 1 h to either DMEPG, VM26, or VP16-213 is shown in Table II. These congeners differ only with respect to the phenyl, thiophene, or methyl groups at the 2-position of the 1,3-dioxane ring formed by the respective aldehyde addition to the glucose moiety. Alkaline elution analysis shows that these groups also influence activity, in that VP16-213 requires a 10-fold higher concentration (1.0 μM) to produce the same break frequency as those produced by DMEPBG and VM26 at 0.1 μM.

These studies demonstrate the existence of a relationship between DNA break formation and chemical structure of the congener at four sites: demethylation at the 4′-position is required for activity; epimerization at the 4′-position potentiates activity; 4-β-D-glucosylation decreases activity; 4,6-O addition to the glucose moiety greatly potentiates activity; and the side group of this addition influences activity.

Kinetics of DNA Break Formation and Repair. The extent of DNA breakage is dependent upon drug concentration, in that the break frequency increases in a nearly linear relationship to VP16-213, VM26, or DMEPBG concentration (Figure 4). A study of DNA break formation resulting from the exposure of cells to 0.5 μM VM for different lengths of

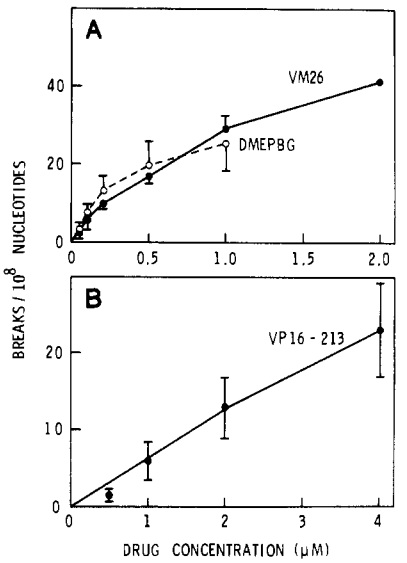


FIGURE 4: Effect of drug concentration on the DNA single-strand break frequency. Cells were exposed to different concentrations of drugs for 1 h at 37 °C and then filtered onto polycarbonate filters. The cells were lysed in the presence of proteinase K and the radioactive DNA was subjected to elution at pH 12.1, as described for Figure 3. Break frequencies were calculated from rad equivalents, which were obtained from the elution slopes. (A) VM26 (●) and DMEPBG (○) and (B) VP16-213 (●). Values represent averages with standard deviation from three separate experiments for each drug.

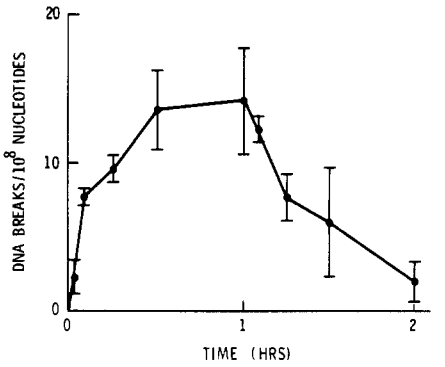


FIGURE 5: Kinetics of DNA break formation and repair. Cells in suspension were incubated with 0.5 μM VM26 at 37 °C for various times up to 1 h or were incubated for 1 h, sedimented by centrifugation, and resuspended in warm, drug-free medium, and incubation was continued for various lengths of time without drug. Radioactive DNA was subjected to elution at pH 12.1 from polycarbonate filters after cell lysis in the presence of proteinase K. Break frequencies were calculated from rad equivalents, which were obtained from the elution slopes. Values represent averages with standard deviation from three separate experiments.

time up to 1 h (Figure 5) reveals that breaks form rapidly upon exposure to drug but their formation plateaus after 30 min. The removal of drug from cells exposed to VM26 for 60 min results in the rapid repair of the drug-induced lesions, an observation first reported by Loike & Horwitz (1976b). After 1 h of repair nearly 80% of the drug-induced breaks are repaired (Figure 5).

Cytotoxic Activities of the Different Congeners. Cytotoxicity was determined by counting cells maintained in flasks in drug-free medium for 5 days after drug treatment and comparing counts with those from untreated controls (Table III). Cell proliferation was markedly inhibited by the antimicrotubule-active congeners in parallel to antimicrotubule activity (see Table I). Glycosylation of DMP resulted in a congener, DMPG, that showed no toxicity at a concentration of 10 μM. Glycosylation of β-peltatin and podophyllotoxin has been shown to nearly abolish antimicrotubule activity of

Table III: Cytotoxicity by the Different Congeners Determined by Inhibition of Cell Growth[a]

congener	% 10^{-5} M	10^{-6} M	10^{-7} M
P	1 ± 1	80 ± 12	
PP	58 ± 40	112 ± 8	
DOP	0	2 ± 3	92
α-PEL	0	12 ± 5	51
β-PEL	0	1 ± 1	22
EP	12 ± 13	106 ± 11	
DMP	3 ± 2	96 ± 22	
DMEP	76 ± 21	95 ± 8	
DMPG	100 ± 0	106 ± 11	
DMEPG	83 ± 30	93 ± 24	
DMPBG	50 ± 8	101 ± 16	
DMEPBG	4 ± 2	42 ± 21	
VM26	5 ± 2	40 ± 20	
VP16-213	62 ± 11	104 ± 21	

[a] Cells in flasks were incubated with different concentrations of the congeners for 1 h at 37 °C and then incubated in a drug-free medium for 5 days, at which time the cells were suspended with trypsin–EDTA and counted. Cell numbers from drug-treated cultures were expressed as percent of control cultures. The values shown represent mean values with standard deviation from at least three separate experiments.

Cytotoxicity

VM, DMEPBG > DMP > VP, DMEP, DMPBG > DMEPG > DMPG

DNA Breakage

VM, DMEPBG > VP, DMEP > DMPBG > DMP > DMEPG > DMPG

FIGURE 6: Comparison of the relative ranking of cytotoxic and DNA breakage activities exhibited by the different congeners of podophyllotoxin. The ranking of congeners for cytotoxic activity was based upon estimated ID_{50} values obtained from probit plots (Long et al., 1982) of data from Table III. Only congeners active in DNA breakage were ranked. DNA breakage activities exhibited by the congeners in Table II were used to rank the congeners in decreasing order with respect to DNA breakage activity.

the highly active unglycosylated drugs (Kelleher, 1977), and glycosylation probably has the same effect on DMPG and DMEPG activity. It is likely that the cytotoxicities displayed by DMEPG, DMPBG, DMEPBG, VM26, and VP16-213 are due to DNA breakage activity rather than antimicrotubule activity, since VM26 and VP16-213 have no detectable antimicrotubule activity at 100 μM (Loike et al., 1978; Brewer et al., 1979). Figure 6 compares DNA breakage activity with cytotoxicity and illustrates the close correspondence between cytotoxicity and DNA breakage activities of the glycosylated congeners. It should be noted that DMP and DMEP also have antimicrotubule activity (Table I) in addition to DNA breakage activity (Table II), but only DMP is more cytotoxic than would be indicated from DNA breakage activity due to strong antimicrotubule activity.

Discussion

Although Loike & Horwitz (1976b) first observed DNA breakage in vivo upon exposure of HeLa cells to VP16-213 and proposed that the drug-induced cytotoxicity was a result of DNA damage, it was not until Wozniak & Ross (1983) and Kalwinsky et al. (1983) demonstrated DNA breakage at low concentrations of VP16-213 using alkaline elution techniques that support was given to the hypothesis. The correlation we find between cytotoxicity and DNA breakage activity of the different congeners of podophyllotoxin adds further support to the hypothesis that DNA damage is the major factor contributing to the cytotoxicity produced by VP16-213 and VM26.

We have confirmed the structure–activity relationship of Loike & Horwitz (1976b) and expanded it to include influences of the groups extending from the 2-position of the 1,3-dioxane ring formed between the acetal linkage and the glucose moiety. The influence of these groups on cytotoxic activity is evident from the work of Keller-Juslên et al. (1971). It was also demonstrated in this work that these groups greatly influence anticancer activity of the drug when the glycoside is glucose but not when it is galactose (Keller-Juslên et al., 1971). The structure–activity relationship can be summarized as follows: (1) demethylation is required for DNA breakage activity; (2) epimerization enhances this activity; (3) glycosylation diminishes breakage activity and abolishes antimicrotubule activity; (4) aldehyde condensation with the OH groups at the C-4 and C-6 positions of the hexapyranose moiety greatly enhances breakage activity, and the structure of the group containing the aldehyde not only influences DNA breakage activity but also influences anticancer activity.

The precise mechanism by which VM26 and VP16-213 produce DNA breakage is unknown. Wozniak & Ross (1983) have suggested that the free hydroxyl group on the 4′-carbon of VP16-213 may be oxidized to a semiquinone free radical intermediate within the cell, which, in turn, may introduce breaks by a free radical mechanism. Another possible mechanism was proposed earlier by Ross et al. (1979) suggesting that the single-strand DNA breaks produced by intercalating drugs are mediated through a nuclear protein, possibly topoisomerase. The structure–activity relationship outlined above is compatible with an enzyme inhibition model. We have recently found that catenation activity of partially purified type II topoisomerase is inhibited by congeners of podophyllotoxin with a structure–activity relationship similar to those reported here for cytotoxicity and DNA breakage (B. H. Long and A. Minocha, unpublished experiments).

It is now evident that VP16-213 and VM26 act by entirely different mechanisms than podophyllotoxin in producing cytotoxicity. VP16-213 and VM26 do not inhibit microtubule assembly (Loike & Horwitz, 1976a) and podophyllotoxin-resistant Chinese hamster ovary cells, which are cross resistant to many other antimicrotubule agents, remain sensitive to VP16-213 (Gupta, 1981) and VM26 (Gupta et al., 1982; Gupta, 1983a). Likewise, cells resistant to VM26 and VP16-213 were not resistant to podophyllotoxin, DMEP, and podophyllotoxin β-D-glucoside (Gupta, 1983b). VP16-213 and VM26 are rather unique in cancer chemotherapy since they are highly effective analogues of a poor antitumor agent (Issell, 1982) with an entirely different mechanism of action from that of the parent compound.

Acknowledgments

We thank David Schaub for his excellent help with the alkaline elution studies and Diane Brattain for conducting the cytotoxicity studies. We are also grateful to Drs. von Wartburg, Stähelin, Bradner, and Suffness for kindly providing the analogues used in this study. Appreciation is extended to Gail Rudder for the preparation of the manuscript.

Registry No. P, 518-28-5; VP16-213, 33419-42-0; VM26, 29767-20-2; α-PEL, 568-53-6; β-PEL, 518-29-6; PP, 477-47-4; DMP, 40505-27-9; DMEP, 6559-91-7; DMPG, 40505-30-4; DMEPG, 23363-35-1; DMPBG, 40553-78-4; DMEPBG, 23363-36-2; EP, 4375-07-9; DOP, 19186-35-7.

References

Brewer, C. F., Loike, J. D., & Horwitz, S. B. (1979) *J. Med. Chem. 22*, 215–221.

Cortese, F., Bhattacharyya, B., & Wolff, J. (1977) *J. Biol. Chem. 252*, 1134-1140.
Ewig, R. A. G., & Kohn, K. W. (1978) *Cancer Res. 38*, 3197-3203.
Grieder, A., Maurer, R., & Stähelin, H. (1974) *Cancer Res. 34*, 1788-1793.
Gupta, R. S. (1981) *Somatic Cell Genet. 7*, 59-71.
Gupta, R. S. (1983a) *Cancer Res. 43*, 505-512.
Gupta, R. S. (1983b) *Cancer Res. 43*, 1568-1574.
Gupta, R. S., Ho, T. K. W., Moffat, M. R. K., & Gupta, R. (1982) *J. Biol. Chem. 257*, 1071-1078.
Issell, B. F. (1982) *Cancer Chemother. Pharmacol. 7*, 73-80.
Kalwinsky, D. K., Look, A. T., Ducore, J., & Fridland, A. (1983) *Cancer Res. 43*, 1592-1597.
Kelleher, J. K. (1977) *Mol. Pharmacol. 13*, 232-241.
Keller-Juslên, C., Kuhn, M., von Wartburg, A., & Stähelin, H. (1971) *J. Med. Chem. 14*, 936-940.
Kohn, K. W. (1979) *Methods Cancer Res. 16*, 291-345.
Kohn, K. W., Friedman, C. A., Ewig, R. A. G., & Igbal, Z. M. (1974) *Biochemistry 13*, 4134-4139.
Kohn, K. W., Erickson, L. C., Ewig, R. A. G., & Friedman, C. A. (1976) *Biochemistry 15*, 4629-4637.
Kohn, K. W., Ewig, R. A. G., Erickson, L. C., & Zwelling, L. A. (1981) in *DNA Repair: A Laboratory Model of Research Techniques* (Friedberg, E. C., & Hanawalt, P. C., Eds.) pp 379-401, Marcel Dekker, New York.
Krishan, A., Paika, K., & Frei, E., III (1975) *J. Cell Biol. 66*, 521-530.
Loike, J. D., & Horwitz, S. B. (1976a) *Biochemistry 15*, 5435-5443.
Loike, J. D., & Horwitz, S. B. (1976b) *Biochemistry 15*, 5443-5448.
Loike, J. D., Brewer, C. F., Sternlicht, H., Gensler, W. J., & Horwitz, S. B. (1978) *Cancer Res. 38*, 2688-2693.
Long, B. H., & Brattain, M. G. (1984) in *Etoposide (VP16-213): Current Status and New Developments* (Issell, B. F., Muggia, F., & Carter, S. K., Eds.) Academic Press, New York (in press).
Long, B. H., Willis, C. E., Prestayko, A. W., & Crooke, S. T. (1982) *Mol. Pharmacol. 22*, 152-157.
Long, B. H., Musial, S. T., Schaub, D. S., & Brattain, M. G. (1983) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 42*, 360.
Misra, N. C., & Roberts, D. (1975) *Cancer Res. 35*, 99-105.
Roberts, D., Hilliard, S., & Peck, C. (1980) *Cancer Res. 40*, 4225-4231.
Ross, W. E., Glaubiger, D. L., & Kohn, K. W. (1978) *Biochim. Biophys. Acta 519*, 23-30.
Ross, W. E., Glaubiger, D. L., & Kohn, K. W. (1979) *Biochim. Biophys. Acta 562*, 41-50.
Savel, H. (1964) *Proc. Am. Assoc. Cancer Res. 5*, 56.
Savel, H. (1966) *Prog. Exp. Tumor Res. 8*, 189-224.
Scher, W., & Friend, C. (1978) *Cancer Res. 38*, 841-849.
Stahëlin, H. (1970) *Eur. J. Cancer 6*, 303-311.
Stahëlin, H. (1972) *Planta Med. 22*, 336-340.
Stahëlin, H. (1973) *Eur. J. Cancer 9*, 215-221.
Wilson, L., & Friedkin, M. (1967) *Biochemistry 6*, 3126-3135.
Wilson, L., & Bryan, J. (1974) *Adv. Cell Mol. Biol. 3*, 21-72.
Wozniak, A. J., & Ross, W. E. (1983) *Cancer Res. 43*, 120-124.

Structures and Dynamics of a Supercoiled DNA†

John H. Shibata, Jess Wilcoxon, J. Michael Schurr,* and Vic Knauf‡

ABSTRACT: Secondary structures of supercoiled (RF) M13mp7 DNA are investigated by time-resolved fluorescence polarization anisotropy, which monitors the magnitude and uniformity of the torsional rigidity. Tertiary structures are monitored by gel electrophoresis. Seven distinct long-lived structural conformers of this supercoiled DNA are identified: four result directly from different replicates of the same standard preparation procedure; one results from an alternate preparation; and two result from irreversible conversions of such forms to daughter products. These seven conformers all exhibit either of two different, but apparently uniform, torsional rigidities, depending upon the buffer type. These and other data imply that two different secondary structures can prevail in this supercoiled DNA and that neither is ordinary B helix. Each conformer also exhibits one of three basic gel mobilities. The observed dual secondary structures, metastability, and hysteresis of this DNA are shown to follow naturally, if the primary function of supercoiling is actually to facilitate remote control of gene activity by site-specific regulatory proteins. A specific model is proposed for gene regulation by protein control of remote junctions between secondary structure domains. The previously inexplicable stimulatory effect of the prmup-1 mutation in the right operator region of the λ repressor is rationalized by certain aspects of this model.

Supercoiling of DNA is required for transcription, replication, recombination, and site-specific binding of certain proteins (Kornberg, 1980; Bauer, 1978; Cozzarelli, 1980; Gellert, 1981). It is widely suspected that the main function of supercoiling is to induce alterations in DNA secondary structure that critically affect its interactions with proteins involved in gene expression (Kornberg, 1980; Bauer, 1978; Cozzarelli, 1980; Gellert, 1981; Wells et al., 1977). Such structural variants may include locally denatured regions (Wells et al., 1977; Dasgupta et al., 1977), cruciform hairpins at inverted repeats (Wells et al., 1977; Lilley, 1980; Panayotatos & Wells, 1981; Mizuuchi et al., 1982; Courey & Wang, 1983), and stretches of Z helix (Klysik et al., 1981; Peck et al., 1982; Nordheim & Rich, 1983a,b). Enhanced susceptibility to S1 nuclease (Wells et al., 1977; Dasgupta et al., 1977; Lilley, 1980; Panayotatos & Wells, 1981), reduced susceptibility to

† From the Department of Chemistry (J.H.S., J.W., and J.M.S.) and the Department of Microbiology (V.K.), University of Washington, Seattle, Washington 98195. *Received July 19, 1983.* This work was supported in part by Grant GM29338-02 from the NIH and Grant PCM-8022822 from the NSF.
‡ Present address: Cal Gene Inc., Davis, CA 95616.

0006-2960/84/0423-1188$01.50/0