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Structure-activity relationships of VP-16 analogues

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Abstract. A total of 27 selected analogues of VP-16 and VM-26 were compared with VP-16 and VM-26 for their relative abilities to stabilize the enzyme-substrate intermediate normally formed between eukaryote topoisomerase II and DNA. This activity was compared with cytotoxicity results obtained using the human colon HCT116 cell line and antitumor results obtained after intraperitoneal injection of mice with murine leukemia P388. The most potent analogues were those containing OH groups (demethyl) in either the 3′ and 4′ or the 3′, 4′, and 5′ positions, the latter being twice as potent as VP-16. VM-26 was only 40% more potent than VP-16 in this assay. It was generally found that the 4′-esters had little activity in vitro, yet were cytotoxic and had antitumor activities. All other analogues with little in vitro activity were not very cytotoxic and had little if any antitumor activity. A very good correlation exists between stabilization of topoisomerase II-DNA intermediates, cytotoxicity, and antitumor activity.

Key words: Topoisomerase II – VP-16 – Analogues – P4 DNA – SAR

Introduction

The effects of molecular biological approaches of studying eukaryote topoisomerases and the effects of different types of compounds on the enzymes have been described by Osheroff and Pommier (both this issue). The present paper describes the effects of different analogues within the same class on topoisomerase II. This communication begins with a brief description of the history of the demethylepipodophyllotoxins and the discovery of topoisomerase II as their primary target. This is followed by studies of DNA intermediate stabilization and potency quantification and closes with a structure-activity relationship summary of the results obtained from our analogue potency studies.

In the 1940s, the antitumor activity of podophyllin resin was described. This discovery led to clinical trials of the major component, which was identified as podophyllotoxin in the 1950s (see Table 1; for reviews see [9, 40, 41]). These trials were stopped because of excessive toxicity and the lack of clinical activity (see [9]). Nevertheless, Sandoz initiated a natural product and analogue program beginning in 1954 in attempts at making a less toxic podophyllotoxin. This effort continued through 1978. During this undertaking an interesting serendipity occurred in that the efforts resulted in compounds displaying a totally different mechanism of action. Stähelin and Von Wartburg [40, 41] have recently published reviews of these efforts.

VP-16 and VM-26 were first synthesized in the mid-1960s [12, 37–39] and VM-26 actually went into clinical trials [27–29] before being licensed, along with VP-16, to Bristol-Myers in 1978. The structure of VP-16 is characterized by the podophyllotoxin ring system, which includes a four-member fused ring system containing a lactone ring and a pendent aromatic ring. VP-16 and VM-26

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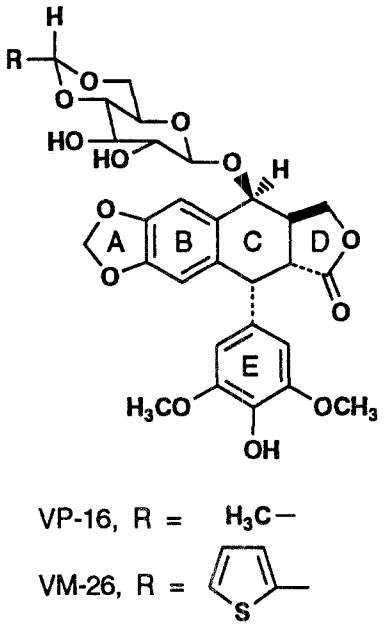
Abbreviations: VP-16, 4′-demethylepipodophyllotoxin-4-(4,6-*O*-ethylidene-β-D-glucopyranoside), Vepesid; VM-26, 4′-demethylepipodophyllotoxin-4-(4,6-*O*-thenylidene-β-D-glucopyranoside), Vumon; MeVP, epipodophyllotoxin-4-(4,6-*O*-ethylidene-β-D-glucopyranoside) or 4′-methyl VP-16; MeVM, epipodophyllotoxin-4-(4,6-*O*-thenylidene-β-D-glucopyranoside) or 4′-methyl VM-26; VPHQ, 3′,4′-didemethylepipodophyllotoxin-4-(4,6-*O*-ethylidene-β-D-glucopyranoside), VP-16 hydroquinone; VMHQ, 3′,4′-didemethylepipodophyllotoxin-4-(4,6-*O*-thenylidene-β-D-glucopyranoside), VM-26 hydroquinone; VPOQ and VMOQ, the orthoquinone forms of VPHQ and VMHQ; DeMeOVP, 3′-demethoxy-4′-demethylepipodophyllotoxin-4-(4,6-*O*-ethylidene-β-D-glucopyranoside); DiDeMeOVP, 3′,5′-didemethoxy-4′-demethylepipodophyllotoxin-4-(4,6-*O*-ethylidene-β-D-glucopyranoside); DiDeMeVP, 3′,4′,5′-tridemethylepipodophyllotoxin-4-(4,6-*O*-ethylidene-β-D-glucopyranoside); desOHVP, 4′-demethoxyepipodophyllotoxin-4-(4,6-*O*-ethylidene-β-D-glucopyranoside); Me, methyl; diMe, dimethyl; MeO, methoxy; DiDeH, didehydro; altro, altrose replaces glucose as the glucoside sugar



VP-16, R = H_3C-

VM-26, R =

Fig. 1. Structure of VP-16 and VM-26

differ from podophyllotoxin in that they are demethylated in the 4′ position, epimerized in the 4 position, and contain a glucose moiety fused to the ring core by a glucoside bond at the 4 OH group (Fig. 1). The glucose is modified by reaction with an aldehyde to form an acetal between the O4 and O6 hydroxyl groups. VP-16 and VM-26 differ from each other only by the group associated with the acetal, whereby VP-16 contains a methyl group and VM-26 contains a 2-thienyl group.

The mechanism-of-action studies for this class of compounds were conducted between 1974 and 1986 and are presently ongoing. These efforts began with the original reports by Loike and Horwitz [14] of DNA breakage being produced in cells. Work by Warren Ross and co-workers in Kurt Kohn's laboratory [32–34] resulted in the discovery of protein-associated DNA breaks produced in cells by DNA intercalators. This, in turn, resulted in reports from the Ross laboratory that VP-16 also produced protein-associated DNA breaks in cells [45] and nuclei [7]. These three important findings led several laboratories to investigate topoisomerase II as the target for VP-16 and VM-26. These efforts resulted in our demonstration of the inhibition of the catalytic activity of eukaryote topoisomerase II by a number of analogues [16, 17, 21] and the demonstration of DNA breakage being induced by VP-16 in the presence of highly purified eukaryote topoisomerase II as reported by Chen et al. [3] and Ross et al. [35]. Several comprehensive reviews of the mechanism of action of the demethylepipodophyllotoxins have been published [4, 6, 15 18, 25].

Bristol-Myers began its analogue program in 1986, and this effort continued through 1990 (Table 1). This program involved the synthesis of approximately 350 analogues, the syntheses of which were divided between the research facilities of the Pharmaceutical Research and Development Division in Wallingford and the Bristol-Myers Research Institute in Tokyo. Responsibility for the synthesis of core-

Table 1. History of demethylepipodophyllotoxin analogue development

Dates	Occurring events
1940s	Antitumor activity of podophyllin described
1950s	Clinical trials of podophyllotoxin
1954–1978	Natural-product and analogue program by Sandoz
1978	Licensing of teniposide and etoposide by Bristol-Myers
1974–1986	Mechanism of action studies
1986–1990	Bristol-Myers analogue program

modified analogues was assigned to the chemists in Wallingford, and chemists in Tokyo assumed responsibility for the synthesis of sugar-modified analogues. Cytotoxicities of the different analogues were determined using sensitive and resistant human carcinoma cell-line models. These were primarily the human colon carcinoma HCT116 cell line [2] and two derived VP-16- and VM-26-resistance variants: HCT116(VM)34, which has increased expression of P-glycoprotein without any alteration of topoisomerase II expression, and HCT116(VP)35, which has decreased expression of topoisomerase II without any change in expression of P-glycoprotein [19]. In vivo studies were conducted with staged intraperitoneal treatment of intraperitoneally growing P388 murine leukemia. Treatments with active compounds resulted in increased life spans, which were expressed as the percentage of treated survivors relative to controls (%T/C) [26]. Advanced in vivo evaluation was accomplished with intraperitoneal treatment of subrenal-capsule-implanted HCT116(VP)35 cells [1, 30].

In vitro evaluations were conducted with emphasis on qualification of the analogue-topoisomerase II interactions, quantification of double-strand DNA breaks, and identification of differences in DNA sequence preferences. Qualification of analogue-topoisomerase II interactions refers primarily to identification of possible differences in inhibition of catalytic activity and differences in ratios between single- and double-strand break production. It was decided that the main emphasis would be on quantification of double-strand DNA breakage.

Materials and methods

Analogues. VP-16 (lot 79F116) and VM-26 (lot 79F117) were obtained from the Bristol-Myers drug repository. MeVP was prepared from VP-16 by glycosylation of podophyllotoxin followed by acetal formation with the appropriate aldehyde as previously described [12]. VPOQ and VMOQ were prepared as described elsewhere [Long et al., submitted for publication]. VPHQ and VMHQ were obtained from VPOQ and VMOQ by chemical reduction. DeMeOVP and desOHVP were synthesized from VP-16, and DiDeMeOVP was obtained through total synthesis [Long et al., submitted for publication]. Many of these analogues had pendent (E) ring modifications, which included 4′-esters and ethers; 4′-H in place of -OH; 3′-OH, -NH_2, or -H in place of -OCH_3; both 3′- and 5′-OH or -H in place of -OCH_3; or 2′-Br in place of -H [31, 36, 42]. Two analogues had modification of the D ring (picro isomer and lactam) [10], one analogue had a partially unsaturated C ring, four analogues had modifications of the A ring [11], and four analogues had substitutions of the 2″ and/or 3″ positions of the sugar and/or altrose in place of glucose as the sugar or methoxymethylidine in place of ethylidene for the acetal [22].

Topoisomerase II purification. Topoisomerase II was purified to near homogeneity from VACO 5 human colon carcinoma cells. The purification procedure will be described elsewhere (B. H. Long and R. R. C. Wang, manuscript in preparation). A total of 200,000–500,000 units of human topoisomerase II devoid of topoisomerase I activity was generally obtained from each preparation; 1 enzyme unit is defined as the minimal activity capable of converting 0.5 μg of knotted P4 DNA by 50% to the unknotted form under the conditions described below.

Assay for topoisomerase II-induced double-strand DNA cleavage. Naturally knotted bacteriophage P4 DNA isolated from tailless capsids was used as the substrate to quantify double-strand DNA break induction by the analogues according to the procedure of Liu et al. [13]. Bacteria and phage strains were kindly provided by Richard Calendar (Department of Molecular Biology, University of California, Berkeley, Calif.). This substrate has also been used to assay quantitatively the inhibition of catalytic activity of eukaryotic topoisomerase II by active agents [8]. Knotted P4 phage DNA (0.5 μg) was incubated with 20 or 5 units of purified topoisomerase II in the presence of 1, 3, 10, 30, and 100 μ*M* of analogue in 20 μl of buffer containing 5 m*M* $MgCl_2$ and 1 m*M* adenosine triphosphate (ATP). After a 30-min incubation period at 37° C, the reactions were stopped by the addition of an ethylenediaminetetraacetic acid (EDTA)-sodium dodecyl sulfate solution containing proteinase K. Any enzyme covalently bound to the DNA at break sites was digested by continued incubation at 37° C for an additional 10 min to reveal the double-strand DNA breaks. The samples were subjected to electrophoresis in 0.9% agarose gels for 15 h, after which the gels were stained by soaking in a 1-μg/ml ethidium bromide solution for 1 h followed by destaining in water for several hours. The different DNA forms were visualized by exposing the stained gels to UV irradiation and the resulting fluorescent DNA bands were photographed. Negatives of the gels were scanned using an LKB model 2222 Ultroscan XL enhanced laser densitometer (Pharmacia LKB Biotechnology, Bromma, Sweden) to quantify the amounts of linear DNA, reflecting stabilized intermediate, in each sample.

We have developed a novel means of expressing the potencies of the different analogues with regard to intermediate stabilization, based on the properties of this enzymatic reaction. Since the stabilized intermediate or protease-cleavable enzyme-DNA complex represents the equilibrium existing between free drug, enzyme, and DNA and the product, i.e., the enzyme – DNA intermediate stabilized by the bound drug [23, 24], then this equilibrium may be expressed in the form of a constant value (k_{app}) defining the relationship between the stabilized intermediate (product) and the reactants.

$$\text{DNA} + \text{Enzyme} + \text{drug} \rightleftharpoons \text{DNA–Enzyme–drug}$$
$$[\text{DNA–Enzyme–drug}] = k_{app}[\text{DNA}][\text{Enzyme}][\text{drug}].$$

When concentrations for DNA, enzyme, and drug are expressed in nanograms, units, and millimolar quantities, respectively,

$$k_{app} = \frac{[\text{DNA–Enzyme–drug}]}{[\text{DNA}][\text{Enzyme}][\text{drug}]} = \text{ng/unit/m}M.$$

This k_{app} value should be different for analogues with different potencies. Also, different enzyme concentrations could be used without affecting the results. In fact, a direct relationship exists between linear DNA production and the concentration of any one component if the other two components are kept invariable and as long as the product (i.e., linear DNA) does not exceed about 20%–30% of the original knotted DNA substrate (B. H. Long and R. R. C. Wang, manuscript in preparation). The analogues evaluated with P4 DNA as the substrate have calculated k_{app} values ranging from 200 to 0, with VP-16 having $k_{app} = 96$.

Assessment of in vivo antitumor activity. P388 ascitic murine leukemia cells were maintained by weekly in vivo passage in DBA/2 and (Balb/c×DBA/2)F_1 hybrid mice. VP-16 or analogues were suspended in carboxymethylcellulose and water supplemented with Tween 80. Various doses of each compound were given in 0.5-ml volumes as intraperitoneal injections on days 1 and 5 following the intraperitoneal implantation of 10^6 P388 cells on day 0. This assay has been described in greater detail elsewhere [Long et al., submitted for publication].

Results

For evaluation of the potencies of newly synthesized VP-16 analogues, several factors needed to be considered. First, human topoisomerase II needed to be purified to near homogeneity; second, the proper DNA substrate for the enzyme had to be selected; and third, a means by which the potencies of the analogues could be expressed in numeric form had to be devised. We had chosen to purify human topoisomerase II from the colon carcinoma cell line VACO 5 [44] because of its short doubling time and its ability to grow in large spinner flasks. A topoisomerase II purification procedure was developed that provided up to 5×10^5 units of enzyme activity void of topoisomerase I activity from about 50 ml of packed cells grown in 40 l of medium. Naturally knotted bacteriophage P4 DNA was selected as the substrate for the topoisomerase II reaction [13]. Quantification of the potencies presented a new obstacle, since quantification of a drug-stabilized DNA-topoisomerase II complex had never been reported. Accurate numeric representation of the potencies of a large number of analogues is important for drug development. This problem was resolved by considering the stabilization of the intermediate as a classic enzyme equilibrium.

In a crude way, we can look at the enzyme reaction as the different reactants (DNA substrate, topoisomerase II, and inhibitor) interacting to form a more stable complex of the normally transient reaction intermediate. The interaction of these three components form an equilibrium that can be broken down into several individual reactions as suggested by Osheroff (this issue). However, these individual reactions can be summed or simplified only by considering the overall reaction. This equilibrium can be expressed by an apparent equilibrium constant K_{app}, which is the ratio of the linear product over the reactants and is expressed as nanograms of linearized DNA per unit of enzyme per millimole of drug (see Materials and methods). For this relationship to be valid, two conditions must be met: first, an equilibrium has to be established, and second, the amount of product produced must be proportional to the amount of enzyme or inhibitor used in the reaction under uniform conditions.

Figure 2 shows the effects of different amounts of human topoisomerase II on the catalytic conversion of the knotted P4 DNA to completely unknotted DNA and on the conversion of circular DNA to linear DNA. The linearization of P4 DNA by DNAase I is also shown for comparison purposes. Three key points should be mentioned with regard to Fig. 2. First, VM-26 is a very poor inhibitor of the catalytic activity of topoisomerase II in that the extent of unknotting produced in the absence of VM-26 as compared with that produced in the presence of 100 μ*M* VM-26 is only about 2 to 3 times greater (comparison of the 1- and 2-unit columns). This lack of pronounced inhibition is significant because VM-26 is a very potent agent for inducing topoisomerase II-mediated DNA breaks in P4 DNA (results not shown). Second, very little enzyme is required to produce double-strand DNA breaks in the presence of 100 μ*M* VM-26, as evidenced by the noticeable presence of linear DNA produced by 0.5 unit of enzyme. Third, there is a proportional increase in double-strand

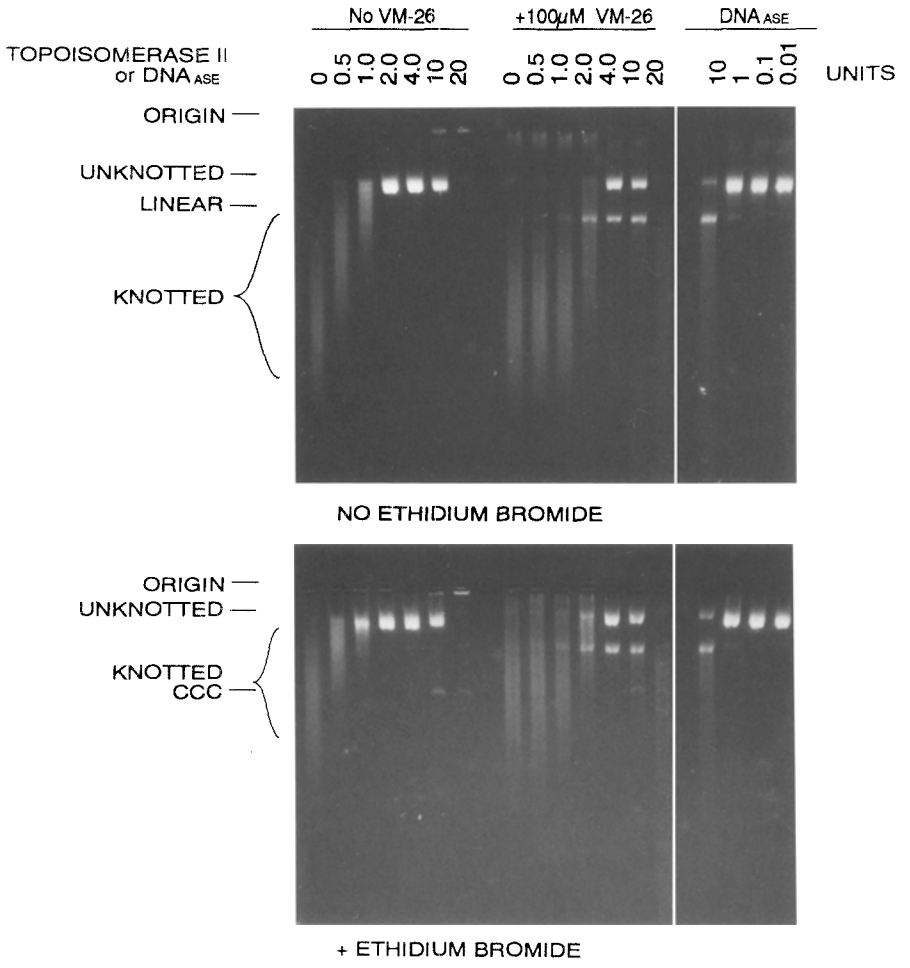


Fig. 2. Agarose gel electrophoresis of P4 DNA incubated with different amounts of human topoisomerase II in the absence and in the presence of VM-26. Each well contained samples of 0.5 μg of knotted P4 with or without 100 μ*M* VM-26 that were incubated for 30 min at 37° C with different units of added human topoisomerase II activity as described in Materials and methods. After the incubation, the reactions were stopped with EDTA-sodium dodecyl sulfate solution containing proteinase K and the DNA-bound enzyme was digested with the protease by incubation for an additional 10 min at 37° C before agarose gel electrophoresis in the absence and presence of 0.5 μg of ethidium bromide/ml. DNAase I treatment of topoisomerase II unknotted P4 DNA was accomplished by adding different units of DNAase just after the addition of 5 units of topoisomerase II. The incubation was conducted in the absence of VM-26

DNA break formation with increasing enzyme concentration (Fig. 2) until the number of multiple cleavage sites on a single molecule become a significant influence in reducing the presence of otherwise intact linear DNA. The induction of multiple cleavage sites in the P4 DNA results in a DNA smear in agarose gels just below the linear DNA band (Fig. 2, 20 units + 100 μ*M* VM-26 lane) and occurs when more than 20%–30% of the DNA is in the linear form. The presence of linear DNA has been shown to reach maximal equilibrium levels within 10 min (results not shown). Thus, all of the criteria have been met to calculate k_{app} values.

One side observation made from this experiment was the presence of a more rapidly migrating band seen only when P4 DNA was incubated with high concentrations of topoisomerase II and when the resulting DNA products were subjected to agarose gel electrophoresis in the presence of ethidium bromide. It should be noted that the migration of unknotted P4 DNA was not influenced by the presence of ethidium bromide in the gel. This resulted because P4 DNA is a circular DNA that is not covalently closed but is maintained as a circle by the presence of cohesive overhanging ends [43]. This band resulted from the presence of covalently closed, circular P4 DNA produced by an inherent ligase activity found associated with pure topoisomerase II. Our findings represent a confirmation of this original observation [5].

Table 2 summarizes the results obtained with 27 analogues and VP-16 and VM-26, the potencies of which are expressed in nanograms of linear DNA produced per unit of enzyme used per millimole of analogue. The most potent analogue was the trihydroxy analogue of VP-16 (3′,4′,5′-tridemethylepipodophyllotoxin ethylidene glucoside; Di-DeMeVP), which was twice as potent as VP-16. This analogue however, is more chemically reactive and is likely to be susceptible to metabolic inactivation. VM-26 was only about 40% more potent than VP-16 under the experimental conditions applied yet was 10 times more potent in cytotoxicity tests, a difference most likely explained by the uptake advantages VM-26 has over VP-16.

In summarizing the structure-activity relationships obtained from these studies, we find that analogues with two or three unblocked hydroxy groups on the pendent aromatic ring of VP-16 are more potent than VP-16 or have a potency equivalent to VM-26 (or slightly lower than that of VM-26 in the case of analogues with these hydroxy groups on the pendent ring of VM-26). Modifications or destruction of the A ring results in analogues with about one-third the potency (5,6-modified analogues). The lactone D ring can be replaced by a lactam, resulting in a slight but significant increase in potency. The picro configuration in the D ring greatly diminishes but does not completely abolish activity directed toward DNA-topoisomerase II intermediate stabilization. However, the coplanar configuration of the molecule resulting from dehydrogenation of the 1C and 2C positions produces an inactive molecule (1,2-dideH-VP). Any substitutions at the 2′ position on the

Table 2. Double-strand DNA breakage reflecting stabilization of enzyme-DNA intermediates by VP-16 analogues compared with their cytotoxic potencies and antitumor activities

Chemical description	Topoisomerase II intermediate stabilization[a]	IC_{50} for HCT116 cytotoxicity[b]	i. p./i. p. P388 Antitumor activity[c]
Highly active:			
DiDeMeVP	182±40	80/ND	>150%/230%
VPOQ	164±30	ND	(3/4)/(2/4)
VPHQ	152±33	35/15	(2/4)/(4/4)
VM-26	133±32	0.4/6	
VP-16 lactam	131±6	60/ND	>185%/295%
MeO-methylidine-VP	(110)	1.1/0.6	>255%/288%
VMHQ	110±22	5/3 (VM)	>(3/4)/(4/6)
VMOQ	99±18	40/3 (VM)	(2/4)/(4/6)
VP-16	96±12	–	–
DeMeOVP	86±13	0.4/0.4	245%/(2/6)
Moderately active:			
5,6-diacetyl-VP	34±4	22/02	>194%/>567%(4)
5,6-thiocarbonyl-VP	31±6	30/0.2	>170%/>510%(3)
2″-F-altro-VP	(27±6)	0.2/0.2	>181%/288%
5,6-desmethylene-VP	21±9	>15/0.3	>165%/320%(2)
DiDeMeOVP	21±8	60/ND	>175%/230%
5,6-diMe-VP	21±5	1.3/0.2	>228%/>567%(4)
3′-amino-VP	15±4	15/15	>228%/(3)
Poorly active:			
desOHVP	9±3	5/0.2	>175%/280%
VP-4′-phosphate	7±5	6.1/0.1	(1)/(1)
picro-VP	5±2	125/ND	>140%/230%
1,2-DiDeH-VP	4±3	>16/0.4	ND
VP-4′-linoleate	3±2	1.1/0.6	>350%/250%
4′-Me-VP	3±1	ND	ND
2′-Br-VP	3±3	26/5.2	>110%/250%
2″-N_3-3″-OAc-altro-VP	(3±1)	3.1/0.3	>140%/288%
VP-4′-octanoate	1±2	0.08/0.2	235%/365%
bis(VP-4′)-sebacoate	1±1	211/22	>130%/260%
bis(VP-4′)-adipate	0±3	92/22	>175%/260%
2″,3″-dibenzoyl-VP	(0±1)	3.8/0.3	>125%/288%

[a] Topoisomerase II intermediate stabilization-potency values are expressed as ng of linear DNA per unit of enzyme per m*M* of drug with standard deviations of results obtained at several different concentrations and at least two different experiments. Parentheses indicate that results are based on only experiment

[b] Cytotoxicity values are median cytotoxic concentrations expressed as μg/ml as compared with the median cytotoxic concentration of VP16, except where (VM) is shown. ND indicates that no data exist

[c] Percent values are ratios for life spans of analogue-treated vs control animals expressed as percentages (%T/C) as compared with the (%T/C) for VP-16-treated vs control animals obtained in the same experiment; values in parentheses are numbers of long-term survivors among 4 test animals treated with analogue as compared with survivors treated with VP-16. ND indicates that no data exist

pendent ring renders the molecule inactive (2′-Br-VP). The inactivity of 2′-substituted molecules is most likely due to the inability of the pendent ring to rotate freely about the bond joining it to the fused four-member ring. However, substitutions at the 3′ position, including relatively bulky substitutions, have little effect other than slightly decreasing the potency of the molecule. The selected sugar-modified analogues are significantly less potent (2″-F-altro-VP, 2″-N_3-3″-OAc-altro-VP, and 2″,3″-dibenzoyl-VP). Of the 26 analogues with antitumor data, 19 were not dosed to toxicity.

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