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Antitumor activity of the two epipodophyllotoxin derivatives VP-16 and VM-26 in preclinical systems: a comparison of in vitro and in vivo drug evaluation*

Peter Buhl Jensen[1], Henrik Roed[1], Torben Skovsgaard[2], Ellen Friche[3], Lars Vindeløv[3], Heine Høi Hansen[1] and Mogens Spang-Thomsen[4]

[1] Department of Oncology, Finsen Institute, Copenhagen
[2] Department of Oncology, University Hospital of Herlev
[3] Department of Internal Medicine, Finsen Institute, Copenhagen
[4] University Institute of Pathological Anatomy, University of Copenhagen, Copenhagen, Denmark

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Summary. The epipodophyllotoxines VP-16 and VM-26 are chemically closely related. VM-26 has been found to be considerably more potent than VP-16 in vitro in a number of investigations. Although the drugs have been known for >20 years, they have not been compared at clearly defined equitoxic doses on an optimal schedule in vivo and it has not been clarified as to whether a therapeutic difference exists between them. A prolonged schedule is optimal for both drugs; accordingly we determined the toxicity in mice using a 5-day schedule. The dose killing 10% of the mice (LD_{10}) was 9.4 mg/kg daily (95% confidence limits, 7.4–11.8) for VP-16 and 3.4 (2.5–4.5) mg/kg daily for VM-26. In vitro, we found VM-26 to be 6–10 times more potent than VP-16 in a clonogenic assay on murine tumors P388 and L1210 leukemia and Ehrlich ascites. This pattern was also demonstrated in a multidrug-resistant subline of Ehrlich selected for resistance to daunorubicin (Ehrlich/DNR+), as it was 30 times less sensitive than Ehrlich cells to both VP-16 and VM-26. Using 90%, 45%, and 22% of the LD_{10} on the same murine tumors in vivo, we found that the effect of the two drugs was equal as evaluated by both the increase in life span and the number of cures. The drugs were also compared in nude mice inoculated with human small-cell lung cancer lines OC-TOL and CPH-SCCL-123; however, they were more toxic to the nude mice and only a limited therapeutic effect was observed. In conclusion, the complete cross-resistance between the two drugs suggests that they have an identical antineoplastic spectrum. VM-26 was more potent than VP-16 in vitro; however, this was not correlated to a therapeutic advantage for VM-26 over VP-16 in vivo

Introduction

For nearly all commonly used cytostatics, there are a number of chemically closely related compounds. To clarify whether therapeutic differences exist between such analogues, comparative analysis of the maximal tolerated dose, toxicological profile, therapeutic activity at equitoxic doses, and antineoplastic spectrum should be performed. The podophyllotoxin derivatives VP16-213 (VP-16, etoposide) and VM-26 (teniposide) represent an example of two compounds that have undergone clinical evaluation since 1970 without clarification as to which one is the most preferable to use. At present VP-16 is used most widely, but due to promising results achieved with VM-26 in vitro [17] and in a recent phase II trial in previously untreated patients with small-cell lung cancer (SCLC) [1], VM-26 has attracted renewed interest.

In a number of in vitro investigations VM-26 was found to be 6–10 times more potent than VP-16 [10, 11, 17, 23], and the present investigations were initiated to evaluate whether the observed difference in potency correlates with a therapeutic advantage for VM-26 as compared with VP-16. Equitoxic dose levels were determined in ND mice, and the effects of VP-16 and VM-26 on murine tumors L1210 and P388 leukemia and Ehrlich ascites were compared in vitro as well as in vivo at equitoxic doses in ND mice. The same treatment schedules were used to examine the effect of the two drugs on two human small-cell lung cancer (SCLC) xenografts in nude mice.

Materials and methods

Drugs. VM-26 was supplied in a solution for infusion containing 10 mg drug, 30 mg benzyl alcohol, 60 mg *N,N*-dimethylacetamide, 500 mg macrogoli ricinoleas, maleic acid to pH 5, and absolute alcohol to give a final volume of 1 ml. VP-16 was supplied in a solution for infusion containing 20 mg drug, 2 mg citric acid, 30 mg benzyl alcohol, 80 mg polysorbate 80, 650 mg macrogol, and absolute alcohol to give a final volume of 1 ml. Both drugs were obtained from Bristol-Myers.

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Offprint requests to: Peter Buhl Jensen, Department of Oncology, Finsen Institute, 49 Strandboulevarden, DK-2100 Copenhagen, Denmark.

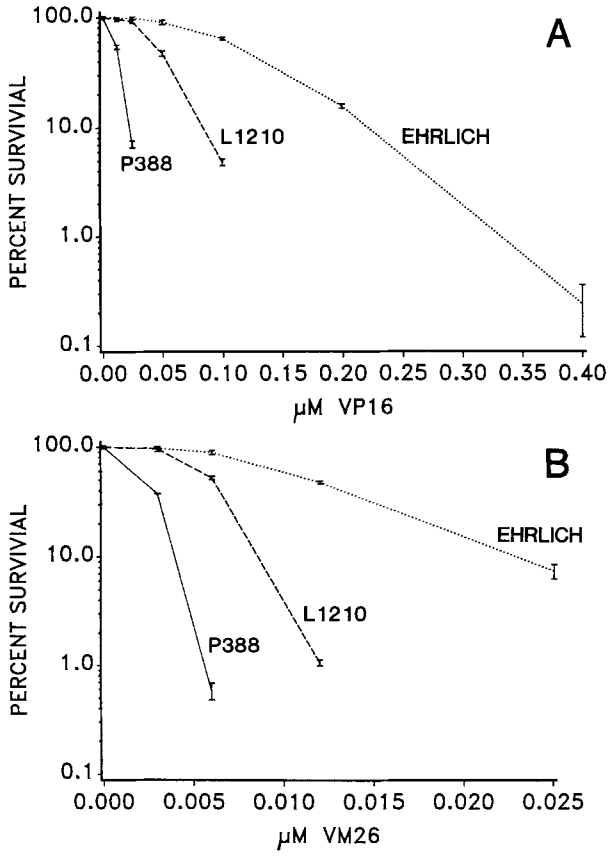

Fig. 1 A, B. Dose-response curves for **A** VP-16 and **B** VM-26 based on the clonogenic assay on Ehrlich (·····), L1210 (– – –), and P388 (———) cell lines. Note the differences in the drug concentrations used. Bars represent the standard error of the mean from triplicate cultures

Cell lines. The serially transplanted murine tumors P388 and L1210 leukemia, Ehrlich ascites tumor, and Ehrlich/DNR+, a classic multidrug-resistant (MDR) subline of Ehrlich selected for resistance to daunorubicin [6, 20], were taken directly from mice. SCLC cell line OC-TOL (also designated GLC-3) was obtained from Dr. Loe de Leij (Groningen, Holland) [14]. CPH-SCCL-123 was established in our laboratory by culturing a pleural exudate from an untreated patient with SCLC. Due to a low and variable plating efficiency, the clonogenic assay was not feasible on this cell line.

Clonogenic assay. Cell survival was assessed by colony formation in soft agar as previously described [17]. Murine ascites tumor was pelleted and washed once in phosphate-buffered saline (150 mM NaCl, 50 mM phosphate, pH 7.2). The cell viability was assessed by dye exclusion in a hemocytometer. A single-cell suspension was plated in soft agar with the desired drug concentrations on top of a feeder layer containing sheep red blood cells. After solidification of the agar, 1 ml medium was added to prevent drying. The number of cells plated were adjusted to obtain approx. 2,000 colonies in the control dishes. After 3 weeks the colonies were counted. The dose reducing the number of colonies to 50% of the control value was computed (LD_{50}) by linear regression analysis as previously described [13].

Toxicity in ND mice. The subacute toxicity of VP-16 vs VM-26 was compared in first-generation ND female mice obtained from DBA male and NMRI female parents (Bommice, Denmark). Mice in groups of ten were treated i. p. once daily for 5 consecutive days. The toxicity of each dose was measured as the percentage of mice that died within 2 months. Control mice were treated with solvent corresponding to the highest dose of the drugs. The LD_{10} with 95% confidence limits was calculated by probit analysis [6].

Table 1. LD_{50} values determined by clonogenic assay

Drug	P388	L1210	Ehrlich	OC-TOL	Ehrlich/DNR+
VP-16	0.0089	0.035	0.083	0.098	3.0
VM-26	0.0014	0.0037	0.0087	0.013	0.29
VP:VM	6.4	9.4	9.5	7.5	10.3

Data are expressed as μM values

Table 2. Relative LD_{50} values determined by clonogenic assay

Drug	EHR/ P388	OC-TOL/ EHR	EHR-DNR+/ EHR	EHR-DNR+/ P388
VP-16	9	1.2	36	337
VM-26	6	1.5	33	207

EHR, Ehrlich ascites tumor; EHR-DNR+, Ehrlich/DNR+

Therapy in ND mice. ND mice inoculated with 1×10^6 P388, 1×10^5 L1210, or 15×10^6 Ehrlich cells were treated i. p. once daily for 5 consecutive days at equitoxic doses, starting 24 h after tumor inoculation. Drug effect was evaluated according to the median survival [9] and the percentage of cures. Tumor-bearing control mice were treated with saline.

Therapy in nude mice. A preliminary report on parts of the xenograft studies has previously been presented [22]. Xenografts were established in nude mice by s. c. inoculation into the flanks of $1-4\times10^6$ OC-TOL or CPH-SCCL-123 cells/0.2 ml culture medium. Serial transplantation of xenografts for the therapy experiments was performed by s. c. insertion of 2-mm-diameter tumor blocks in both flanks of the recipient mice [21]. Treatment was initiated when the tumors had shown growth in six consecutive tumor measurements. Tumors demonstrating no take or growth in less than six growth recordings by the 1st day of treatment were excluded from the experiment. Tumors in mice that died during the posttreatment observation period were also excluded from further analysis. The therapeutic effect was evaluated from rectilinear transformed Gompertz growth curves using a computer program [19] for calculation of the specific growth delay: SGD = (TGD-CGD)/CGD, where TGD and CGD represent the time required for treated and control tumors, respectively, to reach twice the volume recorded on the 1st day of treatment.

Results

In vitro evaluation in murine tumors

Figure 1 shows the results of in vitro testing of VP-16 and VM-26 in murine L1210 and P388 leukemia and Ehrlich ascites cell lines. Table 1 lists the LD_{50} values. For comparison, the LD_{50} values obtained in the OC-TOL [17] and Ehrlich/DNR+ cell lines are shown in Table 2. The results represent a wide range of LD_{50} values, as P388 was 6–9 times more sensitive than Ehrlich and 200–300 times more sensitive than Ehrlich/DNR+. However, VM-26 was 6–10 times more potent than VP-16 in all cell lines tested. This sensitivity pattern demonstrates cross-resistance between the two drugs, and the fact that Ehrlich/DNR+ cells were approx. 30 times less sensitive to both drugs than were parental Ehrlich cells further demonstrate cross-resistance to daunorubicin.

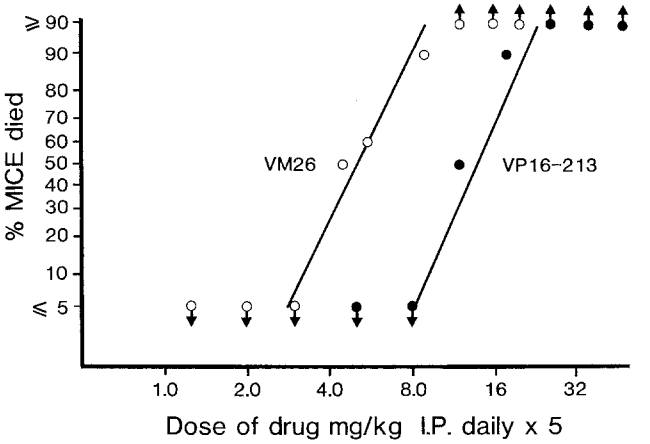

Fig. 2. Long-probit plot of the toxicity of VP-16 and VM-26 in female ND mice. Animals in groups of 10 were treated i.p. for 5 consecutive days. *Arrows pointing downward* indicate that no mice died; *arrows pointing upward* indicate that all mice in the group died

Table 3. The median survival, percentage of cures, and percentage of median increase in life span in mice treated with the indicated equitoxic doses daily for 5 consecutive days

Tumor		Dose	LD_{10} (%)	MS (days)	Cures (%)	ILS[a] (%)
P388	VP-16	8 mg/kg	90	60	81	
	VM-26	3 mg/kg	90	60	72	
	untreated			10		
P388	VP-16	4 mg/kg	45	27	15	200
	VM-26	1.5 mg	45	40	46	344
	untreated			9		
P388	VP-16	2 mg/kg	22	41	13	242
	VM-26	0.75 mg/kg	22	32	13	167
	untreated			12		
L1210	VP-16	8 mg/kg	90	60	55	
	VM-26	3 mg/kg	90	60	70	
	untreated			8		
L1210	VP-16	4 mg/kg	45	23	0	188
	VM-26	1.5 mg/kg	45	22	23	175
	untreated			8		
EHR	VP-16	8 mg/kg	90	41	27	156
	VM-26	3 mg/kg	90	45	27	181
	untreated			16		
EHR	VP-16	4 mg/kg	45	37	18	131
	VM-26	1.5 mg kg	45	36	0	125
	untreated			16		

[a] No value is shown for groups in which >50% of the animals were cured

Results from 7 consecutive experments, each involving 1 untreated control group (15 mice) and 2 groups (11–13 mice) that were treated with equitoxic doses of VP-16 and VM-26. In all, 22 mice were included in each of the groups inoculated with P388 cells that were treated with 22% of the LD_{10}. Surviving mice were killed on the final day of observation (day 60). EHR, Ehrlich ascites tumor; MS, median survival; ILS, median increase in life span

Toxicity in ND mice

None of the control mice died. The corresponding LD_{10} values for VP-16 and VM-26 were 9.4 (95% confidencee limits, 7.4–11.8) mg/kg and 3.4 (2.5–4.5) mg/kg, respectively (Fig. 2).

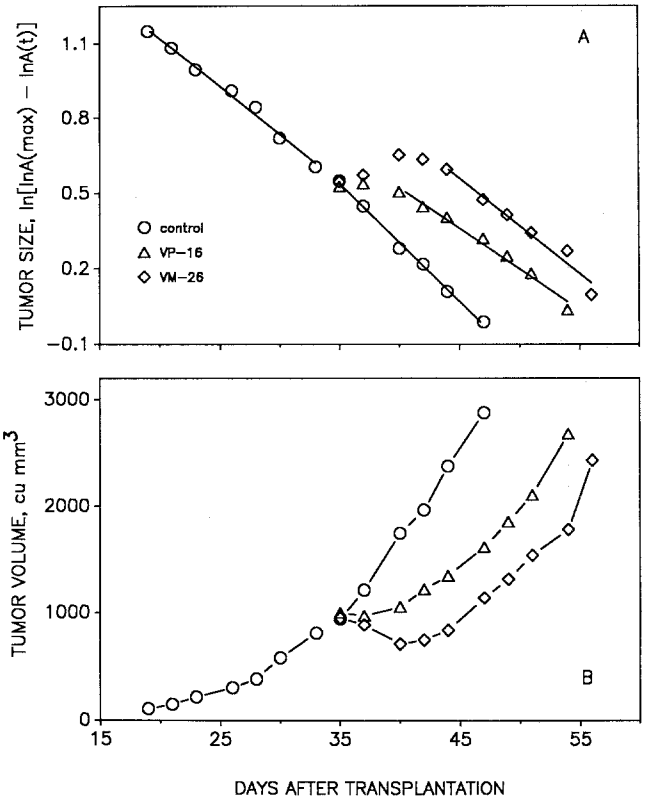

Fig. 3 A, B. Normalized mean growth curves for the OC-TOL SCLC xenograft in nude mice. Tumors were treated with VP-16 or VM-26 at 34–38 days after transplantation. **A** Transformed Gompertz growth curves and best-fit regression lines. **B** Tumor-volume growth curves. ○, control; △, VP-16; ◇, VM-26

In vivo evaluation in ND mice

Table 3 shows the treatment results obtained in ND mice inoculated with Ehrlich, L1210, and P388 cells. The mice were treated with 90%, 45%, or 22% of the LD_{10}. The effect of the two drugs on the tumors varied, with the lowest effect being obtained in Ehrlich ascites tumor and the highest, in P388 leukemia. With respect to cures, apparently VP-16 was most effective in two experiments, VM-26 was most effective in three other experiments, and the effect was identical in yet two others. The increases in life span (ILS) achieved with the two drugs were almost identical in three experiments. Although there was interexperimental variation, the overall result is that VP-16 and VM-26 are equally effective when given in one daily i.p. dose for 5 consecutive days.

In vivo evaluation in nude mice

We found no antitumor effect on SCLC lines OC-TOL and CPH-SCCL-123 grown in nude mice when the animals were treated with 45% of the LD_{10} as defined for immunocompetent mice. At 90% of the LD_{10}, more toxicity was observed in tumor-bearing nude mice than in immunocompetent rodents. As a result of this increased toxicity to nude mice, we had to repeat this experiment several times to obtain a satisfactory number of tumor measure-

ments. Most experiments on OC-TOL tumors showed a trend towards the superiority of VM-26, and in one experiment a sufficient number of mice survived to produce the results shown in Fig. 3. The number of tumors analyzed were 9, 11, and 6 in the control, VP-16-treated, and VM-26-treated groups, respectively. The SGD values were 1.07 and 1.83 for the VP-16- and VM-26-treated groups, respectively. None of the animals included in the control group, died during the experimental period, whereas 3/9 and 4/9 mice died in the VP-16-treated and VM-26-treated groups, respectively. No significant antitumor effect was obtained in CPH-SCCL-123 tumors treated with 90% of the LD_{10}; SGD values were 0.9 and 0.7 for VM-26 and VP-16, respectively.

Discussion

In a previously published study using a clonogenic assay to compare the activity of VP-16 and VM-26 against five human SCLC cell lines, cell-cycle-phase specificity was suggested by the fact that the drugs were considerably more potent with continuous drug exposure than with a 1-h drug incubation [17]. Likewise, the dose-response curves constructed for a 1-h incubation reached a saturation value, whereas those plotted for continuous incubation were exponential. These results were obtained using VP-16 as well as VM-26, indicating that the optimal schedule for both analogues is prolonged administration for several consecutive days [17].

The schedule dependency of VP-16 observed in the clonogenic assay has been demonstrated in vivo in murine leukemia L1210 by Dombernowsky and Nissen [7]. One treatment with an optimal dose (45 mg/kg) gave only a 75% ILS and cured only 13% of the mice, whereas treatment with the same cumulative dose every 3 h for 24 h resulted in an ILS of >650% and cures in 50% of the animals. Recently, a clinical pharmacokinetic study of VP-16 in patients with SCLC demonstrated the superiority of a 5-day schedule over a 24-h schedule; the same cumulative dose resulted in a response rate of 10% and 90%, respectively, and it was suggested that the superiority of the 5-day schedule was related to the maintenance of sufficient, albeit low, plasma drug concentrations over a prolonged period [2]. This study corroborates the results obtained in the clonogenic assay with continuous drug exposure [17]. Although the best schedule is not necessarily the same for the two analogues [18], fractionated treatment with VM-26 is also superior to single-dose therapy [24]; therefore, a 5-day schedule was used for both drugs in the present in vivo investigations.

The antineoplastic effects of VP-16 and VM-26 have been compared in vitro in a number of cell lines [10, 11, 17, 23]. (Table 1). In all of these investigations, VM-26 has been found to be 6–10 times more potent than VP-16. This difference in potency has also been observed on the effect of topoisomerase II-mediated DNA breaks in vitro [15]. Based on these studies, the use of VM-26 should theoretically be more advantageous, as the drug is only 2.5–3 times more toxic than VP-16 (Fig. 2). The antineoplastic effects of VP-16 and VM-26 in vivo have been compared in only a few studies that did not use optimal doses and schedules. In mice inoculated with L1210 cells and treated i.p. on days 1, 4, and 7 with the maximal doses of VM-26 (4.8 mg/kg) or VP-16 (18 mg/kg) (VP-16:VM-26, 3.7:1) that could be given without causing death from delayed toxicity, a comparable effect was achieved (32% ILS) [16].

Some superiority of VM-26 was demonstrated when the two drugs were given at single or repeated doses of 40 or 20 mg/kg (VP-16:VM26, 2:1) to Lewis lung-carcinoma-bearing mice [3]. Likewise, when we treated nude mice bearing the SCLC tumor OC-TOL with 90% of the LD_{10} (VP-16:VM-26, 8:3) in the present study, we found some superiority of VM-26 over VP-16. It is possible that this slight difference in activity is due to differences in the pharmacokinetic properties of the drugs [5]. However, both drugs were considerably more toxic to nude mice than to immunocompentent mice; moreover, although epipodophyllotoxins are among the most effective single agents in the treatment of SCLC, no significant response (SGD, <1) was obtained in CPH-SCCL-123 tumors and only a marginal effect was noted in OC-TOL xenografts. A lack of response to VP-16 by xenografted germ-cell tumors despite the proven clinical effectiveness has also been demonstrated in another laboratory (Schlappack, personal communication).

In contrast to the results obtained in nude mice, we found the effect of the two analogues to be equal when the same doses were given in repeated in vivo experiments on murine P388 and L1210 leukemia and Ehrlich ascites tumors. Both drugs were highly effective at 90% of the LD_{10}; therefore, the experiments were also performed using lower fractions of the LD_{10}. Nevertheless, the therapeutic effects of the drugs were equal at all doses tested, demonstrating that the difference in potency in vivo was corresponded inversely to the difference in toxicity.

Comparison of the in vitro results reveals that OC-TOL, which was selected as one of the most sensitive human cell lines, apparently has a sensitivity in the range of the least sensitive murine line (Ehrlich ascites tumor). Nevertheless, our ability to cure a substantial fraction of the mice inoculated with Ehrlich ascites tumor could suggest activity against the human cell line, thus confirming the clinical experience. These results point to the possible use of a "hybrid sensitivity test": by comparing the sensitivity of SCLC cells with that of murine ascites tumors in the clonogenic assay and combining this finding with the in vivo effect of drugs on the ascitres tumors, we may obtain information that is relevant to preclinical drug evaluation. A similar in vitro-to-in vivo translation has recently been proposed by Double and Bibby [8], who argue for the inclusion of appropriate transplantable mouse-tumor models in "disease-oriented" drug screening [4], as it would enable the ranking of potential anticancer agents in terms of therapeutic index.

In conclusion, we found that VM-26 was 6–10 times more potent than VP-16 in vitro; however, in vivo testing in tumor-bearing mice at equitoxic doses revealed no difference in the antitumor effect on murine tumors in immunocompetent mice and only a small difference in one of the SCLC xenografts tested in nude mice. A persisting

goal of in vitro sensitivity testing is the determination of sensitivity patterns for the identification of drugs that are not cross-resistant to established agents [12]. In the present investigation, the sensitivity pattern of the two analogues was identical, even on the multidrug-resistant line Ehrlich/DNR+. The complete cross-resistance between the two drugs suggests an identical antineoplastic spectrum. Thus, the results indicate that no significant differences between the two drugs should be expected.

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