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# Comparison between VP 16 and VM 26 in Lewis Lung Carcinoma of the Mouse

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**Abstract**—*The antitumoral activity and pharmacokinetics of VP* 16 *and VM* 26 *were comparatively investigated in Lewis lung carcinoma (3LL)-bearing mice. When the two drugs were given at equitoxic doses, in single or repeated treatment, the superior antitumoral activity of VM* 26 *was clear. Compared to VP* 16, *VM* 26 *had a different pattern of distribution, with a larger volume of distribution, a longer elimination half-life time and a lower clearance. The tissue to plasma AUC ratios indicated that VM* 26 *concentrated more in tumor and heart while VP* 16 *gave highest concentrations in liver and intestine. Flow cytometry studies showed that VM* 26 *was more potent than VP* 16 *in causing cell cycle perturbation of 3LL cells growing in primary culture. VM* 26 *displayed cytotoxic activity at a concentration in the medium one-tenth that of VP* 16. *The uptake of VM* 26 *by 3LL cells was* 15 *times that of VP* 16.

## INTRODUCTION

ETOPOSIDE (VP 16–213) and teniposide (VM 26) are two semisynthetic podophyllotoxin derivatives that are effective against several experimental and clinical malignancies [1–8]. There is no evidence that the mechanism of action of the two drugs is different [9]. They both block cells in the premitotic stage and do not act as a spindle poison like the parent podophyllotoxin [10–12]. Despite the fact that these two drugs have been used in clinical practice for many years, there is still no answer to the question of whether they are equivalent or whether there are differences that justify the use of VP 16 for some human tumors and VM 26 for others. Thus experimental and clinical studies to assess differences and similarities are warranted.

Using the model of Lewis lung carcinoma in the mouse, we conducted comparative studies of the antitumoral activity and pharmacokinetics of both compounds. We present evidence that VM 26 is more effective than VP 16 on this murine model and this superiority appears related to more favorable pharmacokinetic properties.

## MATERIALS AND METHODS

*Drugs*

VP 16 and VM 26 were kindly supplied by Dr. G. Lenaz, International Bristol Myers (New York). VP 16 was dissolved in Tween 80 and diluted in saline or growth medium (1/10), VM 26 in DMSO, Tween 80 and saline or growth medium (0.5/1/8.5).

In vivo *experiments*

Male C57BL/6J mice (20 ± 2 g body wt) were used; the animals received an i.m. transplant of $10^5$ viable cells of Lewis lung carcinoma (3LL). For assessment of the antitumoral activity the animals were treated with a single i.v. dose (VP 16 40 mg/kg, VM 26 20 mg/kg) on day 8, or three doses (VP 16 13 mg/kg, VM 26 6.5 mg/kg) on days 8, 11 and 14. One group of animals was killed 25 days after tumor implantation and tumor and metastasis weights were recorded; another group was used to record survival time.

For host toxicity each animal (five mice per group) was weighed every 2 days after treatment and body weight loss was noted [13]. For hematological toxicity blood was taken from the eyes of individual animals (five mice per group) and white blood cell counts were made in a Bürker hemocytometer. Blood cell counts were made in normal and 3LL-bearing mice.

For pharmacokinetic studies mice bearing 25-day-old 3LL were used.

For drug assay HPLC separation after extraction with chloroform was used, according to a method described in detail elsewhere [4, 14].

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In vitro *experiments*

Small tumor fragments were removed aseptically from mice bearing 21-year-old 3LL and washed in a Petri dish with phosphate-buffered saline (PBS) supplemented with antibiotics (penicillin–streptomycin in solution, 10,000 units/ml). Trypsin 1/250 (Difco certified), 0.25% in PBS, was used to obtain a cell suspension after 30 min of incubation at 37°C with continuous shaking. After counting the viable cells by the dye exclusion method with Trypan blue, $2 \times 10^5$ cells/ml were seeded in growth medium in 24-well tissue culture cluster dishes (Nunc) and incubated at 37°C as previously described [3]. Using the concentration of 0.25% of trypsin, at least 95–98% of the cells were viable. Exposure time was 24 hr; at the end of treatment or after 12 hr post-incubation (corresponding to three doubling times), during which cells were maintained in drug-free medium, the wells were emptied, washed once with PBS and the effect of treatment assessed.

The cytotoxicity of VP 16 and VM 26 was investigated by two different methods: (a) as inhibition of thymidine ([$^3$H]Tdr) uptake, adding 0.5 μCi [$^3$H]Tdr, sp. act. 1.9 Ci/mM (Schwarz Mann, Orangeburg) to the wells for the last 1 hr of treatment or recovery. At the end of incubation cells were washed twice with PBS, lysed by 1% sodium dodecylsulfate (SDS) and counted in a toluene-based phosphorus scintillation fluid with a Packard Tricarb 3400 scintillator; and (b) as reduction in the cell count, made using a Coulter Counter Model ZB (Coulter Electronics Ltd, Northwell Drive, U.K.).

*Flow cytometry*

Cells in culture were washed with PBS after VP 16 and VM 26 treatment and directly stained with propidium iodide (P.I.) solution containing 50 μg/ml P.I. (Calbiochem Behring Co., U.S.A.) in 0.1% sodium citrate, 30 μg Nonidet P 40 detergent (Sigma) and 30 μl RNase 0.5 mg/ml in water (Calbiochem Behring Co.) at room temperature for 30–45 min. With this method nuclei were dislodged from the cells adhering to the plastic surface of the tissue culture flasks and entered into suspension without the cells having to be suspended [15].

Flow cytometry analysis was performed on a 30 L cytofluorograph (Ortho Instruments, U.S.A.) detecting the fluorescence pulses in a spectral range between 580 and 750 nm. The coefficient of variation (CV) of the standard (leukocytes from C57BL/6 mouse) was between 1.5 and 2.5%, while in the 3LL cells the CV of the $G_0/G_1$ peak was 3–4% [16]. The percentage of cell cycle phases was calculated by the method of Krishan and Frei [17].

*Drug uptake*

The cell suspension was obtained as described for *in vitro* experiments. Samples of $8 \times 10^6$ viable cells in 1 ml phosphate buffer were incubated at 37°C in a Dubnoff shaker apparatus in the presence of two different concentrations of VP 16 and VM 26. Incubation was stopped by adding 9 ml of ice-cold buffer and the tubes were centrifuged at 1500 rpm for 10 min at 4°C. The cells were washed by adding buffer and centrifuging at 1500 rpm for 10 min and the pellet was gently resuspended in 1 ml buffer. VP 16 and VM 26 content was assessed after separation in HPLC as described elsewhere [4, 14].

*Statistical and pharmacokinetic analysis*

Statistical significance of the differences between treated animal groups was assessed by Student's *t* test. Pharmacokinetic parameters were calculated using the peeling method assuming a two-compartment open model. The areas under the concentration vs time curves (AUC) were measured by trapezoidal integration.

## RESULTS

Table 1 briefly outlines the antitumoral and antimetastatic activity of VP 16 and VM 26 in 3LL carcinoma of the mouse, using two different dose schedules. Better antitumoral and antimetastatic activity was observed in mice treated with divided doses of each drug. After single or repeated doses VM 26 appeared to be more effective than VP 16 on this murine experimental tumor.

The equitoxicity of VP 16 and VM 26 treatments is shown in Fig. 1. In normal mice (panels on the left) both the nadir of WBC and the time of recovery were similar after 40 mg/kg VP 16 or 20 mg/kg VM 26. The weight of the mice was not affected by either drug.

In the case of 3LL-bearing mice, evaluation of the two drugs' leukopenic effects was more difficult because of leukocytosis due to the tumor [18]. Body weight changes were also harder to assess in tumor-bearing animals because they are partially influenced by the antitumor effects of the two drugs, not only by their toxicity. However, in 3LL-bearing mice WBC counts and body weights were similar after 40 mg/kg VP 16 or 20 mg/kg VM 26.

The cytoxicity of VP 16 and VM 26 against 3LL grown in primary culture was investigated by cell counting at the end of 24 hr of treatment and after a period of 72 hr recovery in which cells were maintained in drug-free medium. Figure 2 summarizes five separate experiments expressing the findings as mean percentage of control values. The activity of VM 26 was clearly greater at concentrations of 0.017 and 0.17 μM. At a dose of 1.7 μM VM 26 appeared to be more active than VP 16 only at the end of treatment, whilst 72 hr after 17

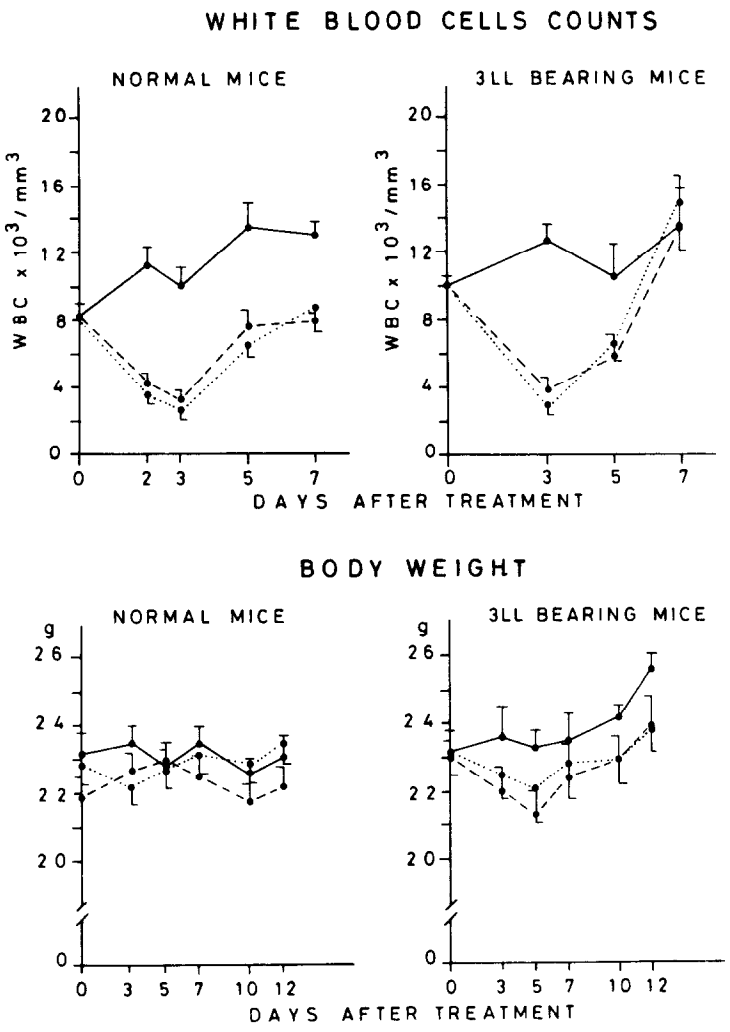


*Fig. 1. White blood cell counts and body weight changes after VP 16 and VM 26 treatment. ——Controls; -----VP 16; .....VM 26.*

*Table 1. Antitumoral and antimetastatic activity of VP 16 and VM 26 in i.m. 3LL-bearing mice*

| Drug and schedule (mg/kg i.v.) | Toxic deaths (%) | Tumor weight† inhibition (%) | Metastases free mice (%) | Survival T/C × 10‡ |
|---|---|---|---|---|
| VP 16 (40, day 8) | 10 | 16 | 20 | 100 |
| VP 16 (13, days 8, 11, 14) | 0 | 51 | 75 | 139 |
| VM 26 (20, day 8) | 0 | 26* | 78 | 132 |
| VM 26 (6.5, days 8, 11, 14) | 0 | 81** | 100 | 160 |

Weight of the primary tumor of control mice was 8.095 ± 0.415 g; weight of pulmonary metastases of control mice was 101 ± 24 mg.

*$P \leq 0.05$ vs VP 16 group; **$P \leq 0.01$ vs VP 16 group by Student's $t$ test.

†Antitumoral and antimetastatic activity was evaluated 25 days after tumor implantation ($10^5$ cells/mouse) ($n$ = 10 mice per group).

‡T/C × 100 = median survival time of treated mice/median survival time of controls × 100. The median survival time of control mice was 25 days.

μM treatment their cytotoxic effects were very similar.

Figure 3 shows the effects of VP 16 or VM 26 on the cell cycle of 3LL cells grown in primary culture, using flow cytometry. VP 16 caused no evident perturbation in the cell cycle distribution at 0.017 μM. Doses of 0.17 or 1.7 μM of VP 16 significantly increased the percentage of cells in SL–$G_2$–M, with

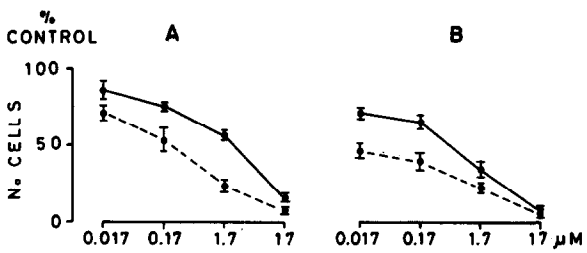


*Fig. 2. Cytotoxicity of VM 26 on 3LL cells in vitro after 24 hr treatment (A) and after 24 hr treatment and 72 hr recovery (B). ——VP 16; -----VM 26. These results are the mean of 5 experiments each consisting of 5 determinations per point.*

a corresponding decrease in the percentage in $G_1$. At 17 μM of VP 16 no $G_1$ peak was seen and the cells appeared blocked in the S phase.

Similar data were observed for VM 26, which also induced an accumulation of cells in SL–$G_2$–M at 0.017 μM. At higher concentrations there was a significant block in the S phase with the disappearance of the $G_1$ peak.

Studies on TdR uptake again showed that VM 26 was more effective at lower concentrations (see Fig. 4). At the highest concentrations both drugs almost completely inhibited TdR incorporation, which is consistent with flow cytometry data.

To try to gain some insight into the reasons for the greater antitumoral activity and cytotoxicity we investigated the distribution of the two drugs in 3LL-bearing mice and the uptake of the two drugs by isolated 3LL cells. Table 2 summarizes the

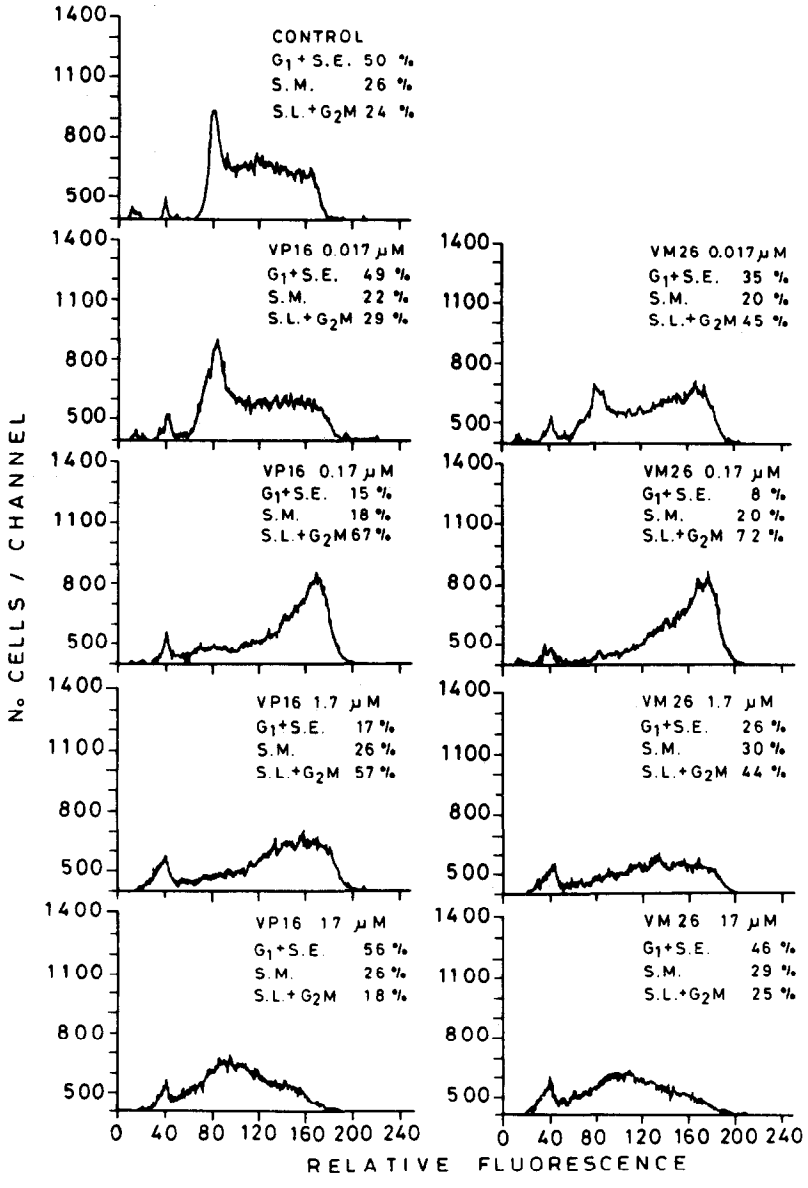


*Fig. 3. Cell cycle phase distribution of 3LL cells after 24 hr of VP 16 and VM 26 treatment in vitro.*

pharmacokinetics in the plasma and primary tumor after single i.v. doses of 40 mg/kg VP 16 and 20 mg/kg VM 26. Plasma peak levels and AUC values were approximately twice those of VM 26, though in the tumor the peak levels and AUC of the two drugs were similar. Elimination of VM 26 from plasma and tumor was slower than VP 16. A lower clearance and a higher volume of distribution of VM 26 were observed.

Table 3 shows tissue/plasma AUC ratios of VP 16 and VM 26 determined for tumor, metastases and some normal tissues of 3LL-bearing mice. For both drugs the ratios in the tumor were lower, probably related to the fact that 3LL tumor is less vascularized than other tissues examined in this study. For both primary tumor and metastases the ratios were higher for VM 26 than for VP 16. In normal tissues VM 26 tended to concentrate somewhat more in heart and VP 16 more in liver and intestine.

Table 4 shows the concentrations of VP 16 and VM 26 in 3LL cells after 30 and 60 min suspension in medium containing 17 μM of each drug. The ratio of intracellular to extracellular concentration was 15 times greater for VM 26 than for VP 16. The same difference was found using 1.7 μM of each drug, but the data are not shown because the reproducibility of the assay at such a low concentration was not completely satisfactory.

## DISCUSSION

This study provides evidence that VM 26 is more effective than VP 16 on the murine 3LL carcinoma. The superior antitumoral activity of VM 26 was shown at equitoxic doses of the two drugs. After repeated treatment lung metastatic involvement disappeared completely in VM 26–treated animals.

Detailed comparative studies of the distribution of VP 16 and VM 26 in 3LL carcinoma bearing mice treated with equitoxic doses of the two drugs (40 and 20 mg/kg i.v. respectively) showed some differences in their distribution. As previously described in patients, the disposition of VM 26 is clearly different from VP 16 [19, 20]. VM 26 has a higher volume of distribution, a longer elimination half-life time and lower clearance than VP 16. The ratios of tissue to plasma AUC, which can give an idea of the distribution in different tissues, showed that VM 26 concentrated more in tumor and heart, and VP 16 in liver and intestine. The higher tumor to plasma AUC ratio for VM 26 was due to a lower VM 26 plasma AUC value, as the tumoral AUC values for VP 16 and VM 26 were very similar. These data suggest that, even at equal drug concentrations in tissue, VM 26 has a greater ability to kill 3LL cells.

It should be stressed, however, that the concentrations of the two drugs were determined in whole tissue and therefore do not represent the drug concentrations at the target sites. In this respect *in vitro* uptake experiments showed that the uptake of VM 26 by 3LL cells was 15 times that of VP 16. A similar difference has already been reported for

*Table 2. Pharmacokinetics of VP 16 and VM 26 in 3LL-bearing mice*

| | Treatment (mg/kg i.v.) | $C_{max}$ (μg/ml or g) | AUC (0→6 hr) (μg/ml × min) | $\alpha T_{1/2}$ (min) | $\beta T_{1/2}$ (min) | $V_d$ (ml/kg) | Cl (ml/kg/min) |
|---|---|---|---|---|---|---|---|
| Plasma | VP 16 (40) | 143 ± 11 | 2431 ± 354 | 1.5 | 33 | 819 | 16.9 |
| | VM 26 (20) | 73 ± 5 | 1480 ± 166 | 2 | 77 | 1390 | 12.0 |
| Tumor | VP 16 (40) | 9.4 ± 1.2 | 1049 ± 296 | — | 57 | N.D. | N.D. |
| | VM 26 (20) | 6.1 ± 1.0 | 999 ± 141 | — | 172 | N.D. | N.D. |

Values are mean ± S.E. of 4 determinations. $C_{57}$BL/6J mice bearing 25-day-old 3LL. $C_{max}$ = drug level at peak; AUC = area under the concentration vs time curve; α, $\beta T_{1/2}$ = half-life; $V_d$ = Volume of distribution β phase; Cl = body clearance.

*Table 3. Tissue/plasma AUC ratios of VP 16 and VM 26.*

| | VP 16 | VM 26 |
|---|---|---|
| Tumor | 0.4 | 0.7 |
| Lung metastases | 1.6 | 1.8 |
| Liver | 11.7 | 4.8 |
| Intestine | 13.8 | 5.7 |
| Kidney | 4.9 | 3.8 |
| Heart | 1.6 | 2.5 |
| Spleen | 1.2 | 1.4 |

VP 16, 40 mg/kg i.v.; VM 26, 20 mg/kg i.v. AUC values were calculated up to 6 hr.

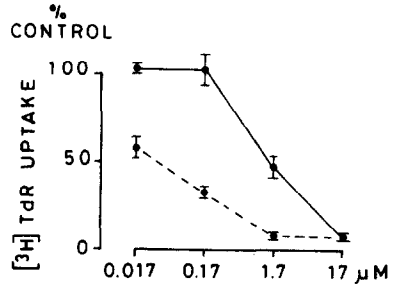


*Fig. 4. Effects of VP 16 and VM 26 on 3LL cells in vitro measured as inhibition of thymidine ([$^3$H]TdR) uptake after 24 hr treatment. ——VP 16; -----VM 26.*

*Table* 4. In vitro *uptake of VP 16 and VM* 26

| | Extracellular concentration (nmol/ml) | 30 min intracellular nmol ± S.E. ($8 \times 10^6$ cells) | 60 min intracellular nmol ± S.E. ($8 \times 10^6$ cells) |
|---|---|---|---|
| VP 16 | 17 | 0.11 ± 0.02 | 0.10 ± 0.01 |
| VM 26 | 17 | 1.6 ± 0.09 | 1.5 ± 0.08 |

$8 \times 10^6$ 3LL cells/ml medium were incubated in suspension at 37°C for 30 or 60 min in the presence of VP 16 or VM 26.

L1210 cells by Allen [21]. The much lower drug levels found in 3LL cells than in L1210 are indicative of some heterogeneity in uptake by different cell types.

The difference in uptake might explain the higher cytotoxic potency of VM 26 as compared with VP 16 observed in different cell types [1, 2] and in this study on 3LL carcinoma cells grown in primary culture. We found that concentrations of 0.017 μM of VP 16 caused no perturbation of the cell cycle and no cytotoxicity, whereas the same concentration of VM 26 produced a significant accumulation of cells in the premitotic stage and was cytotoxic. The arrest of the cell cycle progression through the S phase and the complete shut-down of DNA synthesis was observed at a lower concentration of VM 26 than of VP 16.

The greater cell penetration of VM 26 could be related to its higher lipophilicity, which would facilitate its passage through cell membranes, or to its higher binding to some intracellular component, as already proposed for L1210 leukemic cells [21], where a substantial proportion of VM 26 can be removed by exhaustive dialysis at 4°C whereas the tightly bound drug remains after a variety of denaturing treatments. Recently Ross *et al.* [22] reported that VM 26 was ten times more potent in producing topoisomerase-II-mediated DNA breaks, thus suggesting that VM 26 has a greater affinity for this enzyme than VP 16.

The different distribution of the two antitumoral drugs in normal tissue may have important implications concerning toxicity. In this respect it is interesting that the very high VP 16 concentrations in the liver and the intestine may be in line with the clinically reported VP 16 hepatoxicity [23] and gastrointestinal toxicity [24]. The higher concentrations of VM 26 in the heart fit some observations of VM 26 cardiotoxicity in animals and in patients [25].

In conclusion, this study shows that, at least in 3LL carcinoma, VM 26 has a better therapeutic index than VP 16, probably on account of its more favorable distribution. If the differences in the pattern of distribution of VP 16 and VM 26 observed in 3LL-bearing mice occur in man too, there could be quantitative differences in the antineoplastic activity and toxicity of the two analogs. Clinical studies comparing the efficacy of VP 16 and VM 26 in different tumors are therefore indicated on the basis of the present findings.

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