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Activity of etoposide (VP-16) and teniposide (VM-26) in exponential and plateau phase human tumor cell cultures

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The effects of etoposide (VP-16) and teniposide (VM-26) have been evaluated in human epidermoid carcinoma cells (A431, ME180 and HEp3) grown as exponential and plateau phase cultures. A significant increase in resistance to both these chemotherapeutic agents was observed in unfed plateau compared with exponential phase cells. The large differences in cell killing could not be explained by cell cycle specific toxicities resulting from variations in the cell cycle distributions. Rather the differences in the treatment efficacies probably reflect the 5- to 15-fold increase in the proportion of quiescent cells measured in the plateau phase cultures. These findings suggest that non-proliferating cells in tumors may be preferentially spared in treatments utilizing VP-16 and VM-26.

Key words: Etoposide, proliferation, quiescent, teniposide.

Introduction

Etoposide (VP-16) and teniposide (VM-26) are two important antineoplastic agents used against a variety of tumors including testicular and small cell lung cancers, lymphoma, leukemia and Kaposi's sarcoma.[1,2] While the precise mechanism of action of these drugs has not been fully elucidated, the induction of DNA damage,[3,4] perhaps resulting from the capacity of the drugs to interfere with mammalian topoisomerase II,[5,6] has been strongly implicated.

The influence that tumor cell heterogeneity may have on overall tumor response to these anticancer drugs has as yet not been well characterized in human solid tumors. Such heterogeneity may arise *in vivo* because of intrinsic cellular or extrinsic microenvironmental factors. For example, nutrient deprivation and differences in the local milieu of growing tumor cells can cause the formation of subpopulations with distinct proliferative states. These may include a cycling or proliferative subpopulation (P cells), a non-cycling or quiescent subpopulation (Q cells) and a non-proliferating subpopulation destined for death. The P and Q cell subpopulations hold considerable interest since they manifest different inherent sensitivities to a variety of treatment modalities including radiation[7,8] and anticancer drugs.[9,10] In the case of chemotherapeutic agents, depending on the drug and tumor model, therapeutic efficacy may be greater, less or equivalent in P and Q cell populations.[9–13]

With respect to the epipodophyllotoxins, HeLa and mouse leukemia L1210 cells treated with VP-16 failed to demonstrate dramatic proliferation state dependent cell killing.[12] In contrast, studies with a relative small number of normal rodent and human cell lines have shown that different growth conditions could modulate markedly the efficacy of VP-16.[12,13] Similarly we have reported previously that the susceptibility of human carcinoma HEp3 cells to VP-16 was strongly influenced by the mode of growth at the time of the treatment.[11] In the present paper, we have extended those investigations to include two other epidermoid carcinoma cell lines (ME180 and A431) as well as the structurally related anticancer agent VM-26.

Materials and methods

Cell culture and treatment conditions

Three human epidermoid carcinoma cell lines: A431,[14] ME180[15] and HEp3[16] were used in all experiments. Cells were grown as monolayer cultures either in F-12 medium [supplemented with

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10% (ME180) and 20% (HEp3) fetal calf serum (FCS)], or in a 1:1 solution of F-12 and DMEM media plus 10% FCS (A431). Exponential phase cells were prepared by inoculation 3×10^5 cells (ME180, A431) or 8×10^5 HEp3 cells into 75 cm^2 flasks, incubating them at 37°C in an environment of 5% CO_2:95% air and harvesting them 3 (ME180 and A431) or 2 (HEp3) days later. Fed plateau phase cultures were obtained by incubating 1×10^5 (A431 and ME180) and 6×10^5 (HEp3) cells for 10 (A431), 9 (ME180) or 6 (HEp3) days with daily feedings starting from days 6, 5 and 3, respectively. Unfed plateau phase cultures were incubated in a manner similar to fed plateau phase cultures but without the additional feedings. Unfed plateau cells were harvested after 7 (A431 and ME180) or 6 (HEp3) days. For treatment, 2×10^7 cells were suspended in 10 ml of their respective media in a type I spinner vial[17] at 37°C and gassed with 5% CO_2:95% air during the treatment period. After drug exposure, various dilutions of cells were plated into 60 mm dishes, kept at 37°C in a 5% CO_2:95% air environment and cell survival was determined 10–14 days later.

Drugs

VP-16 (Bristol-Myers) was diluted from stock solutions immediately before use in 0.9% NaCl solution. VM-26, kindly provided by Dr SL Kelley (Bristol-Myers, Wallingford, CT), was initially dissolved in the same organic solvent used for VP-16 preparation, and then diluted to a final concentration using 0.9% NaCl. Drug exposures were for 1 or 4 h.

Autoradiography

Monolayer cells were labelled with [^{3}H]thymidine (final activity 0.5 μg/ml) for 24 h, trypsinized, centrifuged onto a glass slide and fixed with 70% ethanol. The slides were dipped in NTB-3 nuclear emulsion (Kodak, Rochester, NY), incubated in the dark at 4°C for various lengths of time and scored for labelled cells using a microscope. At least 300 cells were counted per slide and labelling index (LI) values were determined. Background grains were less than 5 grains per nucleus.

Flow cytometry

FCM analysis was used to determine the percentage of cells in the G_1, S and G_2 + M phases of the cell cycle. Briefly, as has been previously described, [18] cells fixed with 70% methanol were incubated with RNase (30 min) and stained with propidium iodide (15 min). FCM measurements were made with an EPICS V flow cytometer (Coulter Electronics, Inc.) and DNA histograms were analyzed using the Cytology program.

Centrifugal elutriation

Single cell suspensions were elutriated in ice cold medium, using procedures described in detail elsewhere.[18,19] Briefly, fractions were collected at each decrement of the rotor speed (constant flow rate), counted and size distributions calculated using a Coulter counter Channelyzer (Coulter Electronics, Hialeah, FL). The different cell subpopulations then were exposed to VP-16 for 1 or 4 h and cell survival was determined as described above.

Results

The effect of VP-16 and VM-26 treatments on the clonogenic cell survival of human squamous carcinoma cell lines (A431, HEp3 and ME180) maintained in different growth conditions was investigated. As previously shown for HEp3 cells,[11] ME180 and A431 unfed plateau phase cells were found to be markedly more resistant to VP-16 than were exponential cultures (Figure 1). Fed plateau phase cells demonstrated only a slight change in sensitivity compared to their unfed counterparts.

Increasing the length of exposure to VP-16 from 1 to 4 h produced a higher kill in both exponential and plateau phase ME180 cells, but had less of an effect on A431 cells (Figure 2). However, irrespective of the exposure time, for both cell lines the difference in cell kill between the two modes of growth (exponential and plateau) remained.

For comparison to VP-16, the effect of VM-26 on human tumor cells also was evaluated. Figure 3 shows the response of exponential as well as fed and unfed plateau cultures of HEp3, A431 and ME180 cells to treatment with a range of doses of VM-26. Like VP-16 (Figure 1), VM-26 was virtually ineffective at killing plateau phase human tumor cells. In addition, once again similar to the results observed with VP-16 (Figure 2), the efficacy of VM-26 could be enhanced by increasing the drug exposure time from 1 to 4 h (Figure 4).

Since VP-16 and VM-26 differ in their molecular

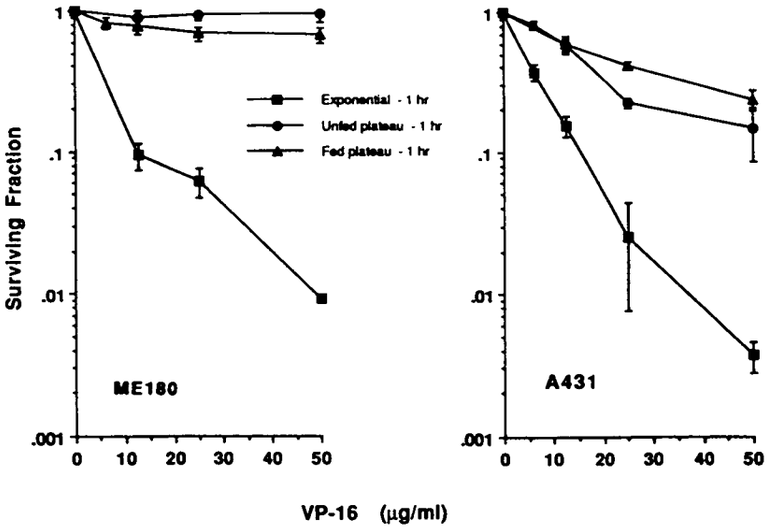

Figure 1. Dose–survival curves of exponential, fed and unfed plateau phase cells treated with VP-16 for 1 h. Data are the mean ± SD of four experiments.

weights, a comparison of the activities of these two agents was made under conditions where drug doses were expressed on a molar basis. The results (Figure 5) showed that VM-26 was approximately 5 to 10 times more potent than VP-16 against two of the cell lines (HEp3 and A431). However, both exponential and plateau phase ME180 cells demonstrated similar sensitivity to VM-26 as to VP-16.

One possible explanation for the large differences in drug efficacies observed between exponential and plateau phase cells (Figures 1–5) is that the results were a consequence of changes in cell cycle distributions coupled with phase specific cell killing by VP-16. The analysis of the cell cycle distributions of exponential and plateau phase cells by flow cytometry did indicate differences between the various cell cultures (Table 1). As expected plateau

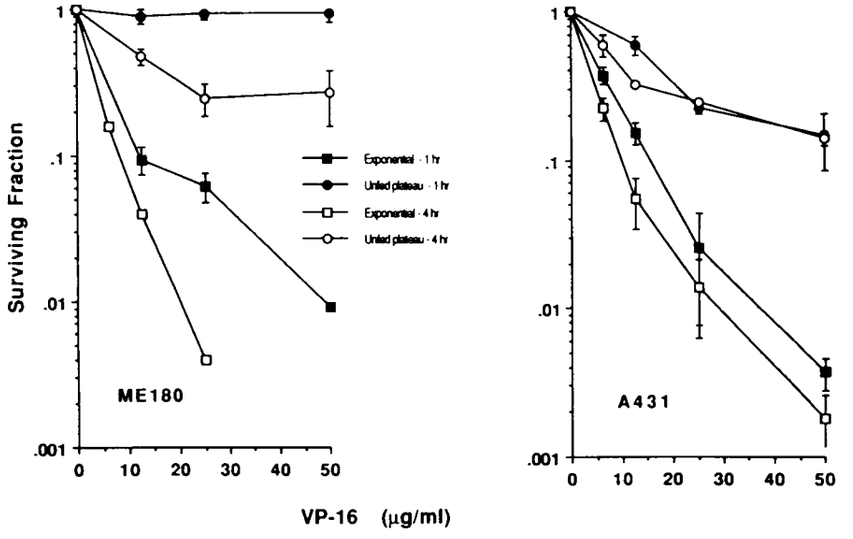

Figure 2. Dose–survival curves of exponentially growing and unfed plateau phase cells treated for 1 or 4 h with VP-16. Data are the mean ± SD of two to four experiments.

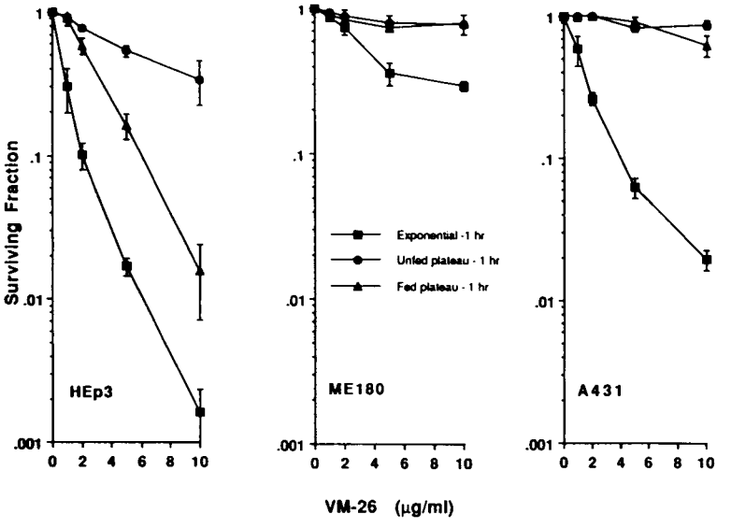

Figure 3. Dose–survival curves of exponentially growing and plateau phase cells treated for 1 h with VM-26. Results are the mean ±SD of two to four experiments.

phase cells (both fed and unfed) showed increases in the proportion of G_1 DNA content cells and a reduction in the proportion of cells with S DNA content. However, these changes were by less than a factor of 2. To evaluate the inherent cell cycle specific toxicity of VP-16, centrifugal elutriation was used to isolate cells in the various cycle phases from asynchronous exponential phase cultures. The isolated cell subpopulations then were exposed to VP-16 and cell survival was assessed (Figure 6). For all three cell lines studied, the resultant age responses indicated only small differences in cell killing (factors of 2–3) between the most sensitive and resistant phases of the cell cycle. Taken together, these observations make it unlikely that changes in the cell cycle distributions are responsible for the large differences in cell killing observed when exponential or plateau phase tumor cells are treated with VP-16.

An alternative explanation for the differences in

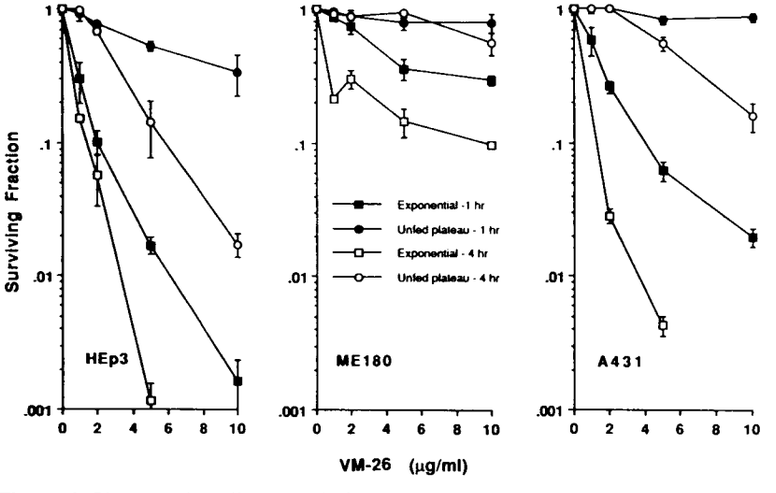

Figure 4. Clonogenic cell survival of exponential or plateau phase HEp3, ME180 and A431 cells treated with VM-26 for 1 versus 4 h. Data are the mean ±SD of two to four experiments.

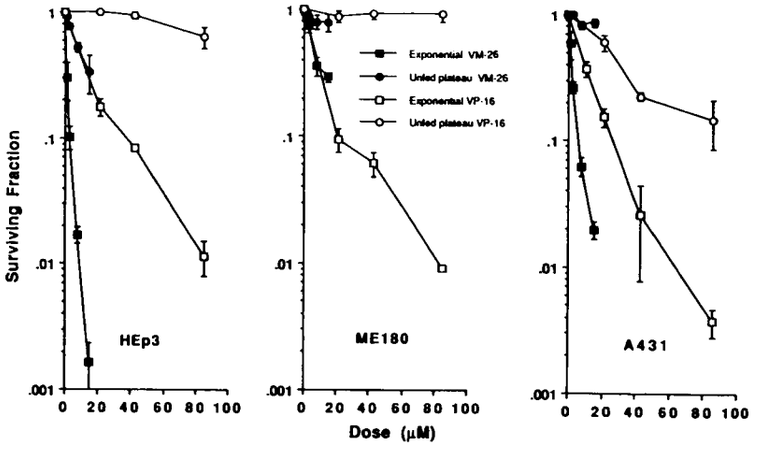

Figure 5. Dose–survival curves of exponentially growing and plateau phase cells treated for 1 h with VM-26 or VP-16. Data are the mean ± SD of two to four experiments.

VP-16 and VM-26 activities seen between exponential and plateau phase (fed and unfed) cells is that the drug resistance seen in plateau phase cell cultures is a consequence of a change from an actively growing to a resting cell population. Experiments therefore were undertaken to determine the proportion of proliferating and non-proliferating cells in cultures under the various growth conditions. Cells were labelled for a 24 h period with [^{3}H]thymidine and the proportion of Q cells were determined (Table 2). The results indicated about a 5- to 15-fold increase in the proportion of quiescent cells in the plateau versus exponential phase cultures for all three cell lines investigated. Unfed plateau phase HEp3 and ME180 cell cultures in particular were composed mostly of quiescent cells.

Table 1. Percentage of cells in various cell cycle compartments (determined by flow cytometry) for human HEp3, ME180 and A431 cells in exponential or plateau phase[a]

Cell line	Type of culture	G_1	S	G_2 + M
HEp3	exponential	58	28	14
	fed plateau	71	18	11
	unfed plateau	75	13	12
ME180	exponential	42	42	16
	fed plateau	56	35	9
	unfed plateau	60	26	14
A431	exponential	51	37	12
	fed plateau	75	18	7
	unfed plateau	77	18	5

[a] Data are the mean of four experiments.

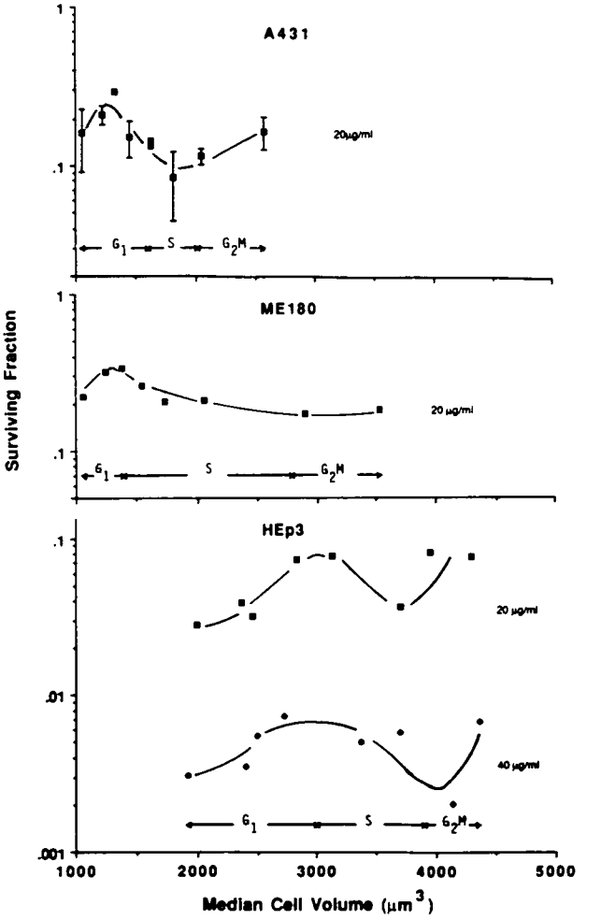

Figure 6. Age response of human epidermoid carcinoma HEp3, ME180 and A431 cells treated with 20 or 40 μg/ml VP-16. Results are the average of two to four experiments.

Table 2. Percentage of quiescent cells in various nutrient conditions determined by 24 h continuous labelling with [^{3}H]thymidine

Cell line	Type of culture	Percentage unlabelled cells[a]
HEp3	exponential	18 ± 4
	fed plateau	94 ± 2
	unfed plateau	99 ± 1
ME180	exponential	20 ± 1
	fed plateau	95 ± 2
	unfed plateau	98 ± 1
A431	exponential	3 ± 2
	fed plateau	49 ± 1
	unfed plateau	52 ± 5

[a]Results are the mean of three experiments ±SD.

Further support for the notion that such a change in cell proliferation state could impact the efficacy of VP-16 comes from the data illustrated in Figure 7. The results indicate that extending the culturing period of ME180 cells from 3 to 7 days leads to both a gradual increase in resistance to VP-16 and a concomitant increase in the proportion of quiescent cells.

Discussion

The effect of VP-16 and VM-26 on three human epidermoid carcinoma cell lines has been evaluated under different growth conditions. At equimolar concentrations, VP-16 and VM-26 previously have been compared in clonogenic assays in the following cell lines including P-875, HeLa, CCRF-CEM, HN-1, CHO and SCLL.[20-24] In all these investigations, VM-26 has been shown to be about 10 times more potent than VP-16. Similar results were observed in the present study for HEp3 and A431 treated with these agents. However, ME180 cells were found to be essentially equally sensitive with both VM-26 and VP-16 (Figure 5).

For the three cell lines investigated, increased resistance to both VP-16 and VM-26 was observed when unfed plateau phase cultures were compared to exponentially growing cells (Figures 1–5). This finding is similar to those of other investigations, which have shown that different growth conditions may modulate markedly the susceptibility of rodent and human cell lines to antineoplastic drugs.[12,25] Under the present treatment conditions, variations of the cell cycle distributions appear not to be sufficient to explain the differences in drug activities. Although VP-16 is a cell cycle phase-specific drug according to the classification of Bruce *et al.*,[26] age–response curves obtained in the present investigations indicated only small differences in cell killing when cells in the various cell cycle phases were treated with this agent (Figure 6). Alternatively the presence of a significant proportion of Q cells in the plateau phase cultures could be a key

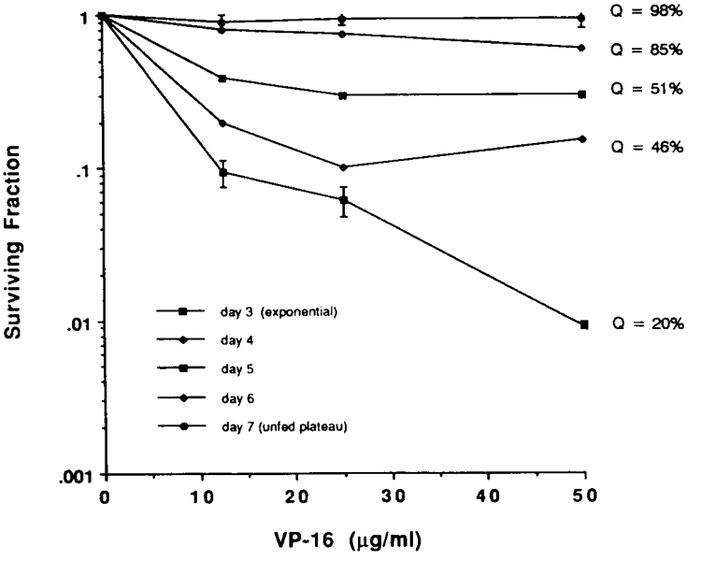

Figure 7. Survival of ME 180 cells treated with VP-16 as day 3 (exponential) to day 7 (plateau) phase cultures. VP-16 exposure was for 1 h. Data are the results of one to four experiments.

factor in determining drug sensitivity (Table 2; Figures 1–4 and 7).

Cell quiescence alone does not, however, appear to be the sole factor responsible for the results observed in the present investigations. Firstly, as the dose or exposure time of VP-16 or VM-26 was increased, cells were killed even in unfed plateau phase cultures containing more than 95% Q cells (Figures 2 and 4; Table 2). Secondly, fed plateau phase cultures which possessed similar proportions of Q cells as their unfed counterparts (Table 2) nevertheless could be more sensitive to the action of VP-16 and VM-26 than the unfed plateau phase cells at least in some cell lines (Figures 1 and 3). Taken together these data imply that, while a quiescent state may readily infer treatment resistance to VP-16 and VM-26, other factors may also contribute to the ultimate sensitivity of tumor cells to these agents.

One key factor in the observed differences in efficacies of the epipodophyllotoxins between exponentially growing and plateau phase cell cultures may be the activity or level of the nuclear enzyme DNA topoisomerase II. This enzyme has been recognized as playing a critical role in the mechanism of action of several clinically useful anticancer drugs, including both intercalating agents such as adriamycin and non-intercalating agents such as VP-16 and VM-26.[27–29] By a mechanism which is still unclear, these drugs stabilize the cleavable complex found between topoisomerase II and DNA, resulting in increased DNA scission and concomitant inhibition of the rejoining reaction.[30] In most studies this has been experimentally expressed as the formation of protein-associated DNA breaks when assayed by the alkaline elution technique.[12,31,32] Although both topoisomerase II activity (measured by the induction of lesions) and drug sensitivity have been shown to vary as a function of the cellular proliferative state, this may not be a consistant finding for all cell lines.[12,32–34]

In the present investigations, the activity of topoisomerase II was not measured. Nevertheless, it seems likely that proliferation state associated changes in topoisomerase II activity may play a role in the relative drug resistance seen in unfed plateau phase cell cultures compared to cells in exponential growth. Further, it is conceivable that differences in the nutritional states of unfed and fed plateau phase cultures could lead to differences in the activity of this enzyme. Thus although fed and unfed plateau phase cell cultures contain approximately equivalent proportions of quiescent cells (Table 2), differences in topoisomerase II activities in these two populations might account for the greater sensitivity of the fed plateau cells to VP-16 and VM-26.

Conclusion

As a tumor grows, the vascular development often cannot keep pace with the rapid neoplastic cell proliferation resulting in heterogeneous tumor cell populations with respect to proliferation and nutritional state. The present findings suggest that cells existing in areas of poor nutrition and quiescent states may be particularly refractory to the action of VP-16 and VM-26.

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