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Flow-Cytometric Analysis of DNA Distribution after VP16-213 Treatment of Lewis Lung Carcinoma

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Summary. *The two dosage schedules of VP16 that gave the least and the greatest efficacy in Lewis lung carcinoma of the mouse were selected for evaluation of the cytokinetic effects observable in vivo at different intervals after treatment (schedule A: 40 mg/kg IV, on day 8 after transplant; schedule B: 13 mg/kg IV, repeated on days 8, 11, and 14 after transplant).*

After the single dose and after each repeated dose there was a marked increase in the percentage of cells in the LS-G_2-M phases, with a corresponding decrease in the percentage of cells in G_0-G_1. The number of neoplastic tetraploid cells compared with normal diploid cells in the tumor was reduced after the single IV dose, and more markedly so after repeated doses.

This study suggests that the more marked delay of cancer cell growth and greater effectiveness observed with schedule B is related to repeated blockade of the LS-G_2-M phases.

Introduction

VP 16-213, or 4′-demethylepipodophyllotoxin ethylidene-β-D-glucopyranoside, is a semisynthetic podophyllotoxin derivative that shows marked antitumoral activity in several rodent [14] and human tumors [2, 8, 9].

In contrast to podophyllotoxin, VP 16 does not appear to act by inhibiting microtubule assembly, but by causing single- or double-strand breaks in DNA [12, 16], so that it arrests the cells in a premitotic phase.

This cytokinetic effect has been observed on different cell types exposed to VP 16 in vitro [4, 7, 11], whereas scant in vivo information is available on administration to tumor-bearing animals or patients. In vivo studies are particularly warranted, considering that the drug concentrations and exposure times of tumoral cells may be different from the in vitro situation, and also because of the reported presence of metabolites [1, 5], possibly with different effects on the cell cycle.

In this report we describe the effects of VP 16 on the cell cycle evaluated by flow cytometry in Lewis lung carcinoma (3LL) in vivo. After investigating the activity of VP 16 in different dosage schedules in this experimental tumor we selected the two schedules which gave the least and greatest efficacy to assess any correlations between cell cycle perturbations and antitumor activity.

Materials and Methods

Animals and Tumor. C57Bl/6 male mice (20 ± 2 g body weight), obtained from Charles River, Italy, were used for these experiments. Syngeneic Lewis lung carcinoma (3LL) cells (10^5) maintained in the same strain by IM passage every 15 days were transplanted IM.

Drugs. VP 16 was kindly supplied by Dr G. Lenaz, Bristol Myers, New York (NY, USA). The drug was dissolved in Tween 80 and saline (1 : 10) and injected IV in the tail vein.

Effects and Hematological Toxicity. For evaluation of the antitumor activity one group of mice was killed on day 25 after tumor implantation and tumor weights were recorded. Another group was used to record the survival time. The median survival time was calculated as described by Geran et al. [6]. Blood cell counts were made before and every 48 h after VP 16 treatment. Blood was taken from individual animals (5 mice per each group). Cell counts were taken in a Bürker hemocytometer.

Preparation of Tumor Samples for Flow Cytometry Analysis. The tumors were removed, weighed, and washed in a petri dish with phosphate-buffered saline (PBS), after which fragments were minced with scissors to separate the vegetative part, which was used for cell analysis, from the necrotic part. The cell suspension for analysis by flow cytometry was obtained by the method of Starace et al. [15]. Tumor fragments were forced through a 18GXl½ needle and resuspended in Hank's solution maintained at 4° C. The cells were stained with propidium iodide (P.I.) (Calbiochem Behring CO, USA) by adding 3 ml P.I. solution (50 µg/ml in sodium citrate 0.1% plus 30 µl Nonidet P 40) to 200–300 µl cell suspension stored at 4° C for 20–30 min before analysis. The suitability of the preparation, the specificity of staining, and the absence of aggregates were checked by fluorescence microscopy before the sample was run. Leukocytes from C57Bl/6 mice were used as standard.

Cytofluorimetric analysis was performed using a 30L Cytofluorograph (Ortho Instruments, USA). The fluorescence pulses were detected in a spectral range between 580 and 750 nm (to exclude the overlapping region of excitation and emission spectra or unbound P.I.) and integrated. To calculate the percentage of cells in cell cycle phases, we used the method of Krishan and Frei [10].

The coefficient of variation (CV) of the leukocyte standard was between 1.5% and 2.5%, while that for the G_0-G_1 phase of 3LL cells was between 4% and 6% CV. The results are the means of two experiments in groups of four animals. Each

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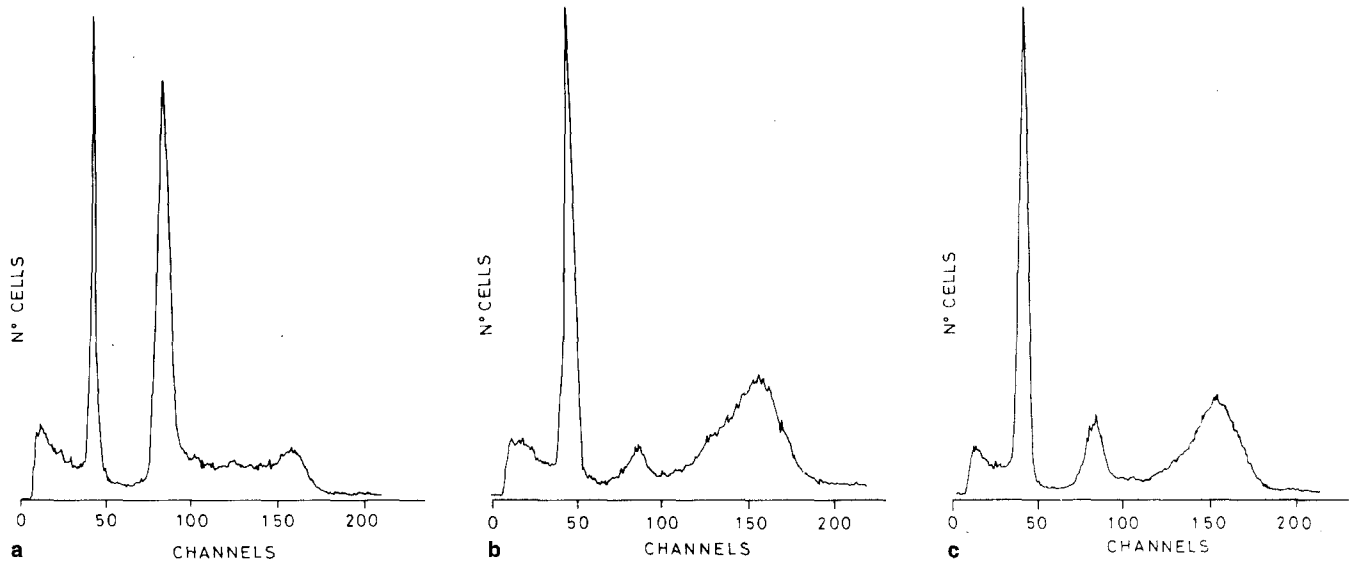

Fig. 1a–c. DNA histograms of Lewis lung carcinoma (3LL) cells without treatment (**a**), 24 h after VP 16-213 treatment at dose of 40 mg/kg IV (**b**), and 24 h after VP 16-213 treatment at 13 mg/kg IV (**c**)

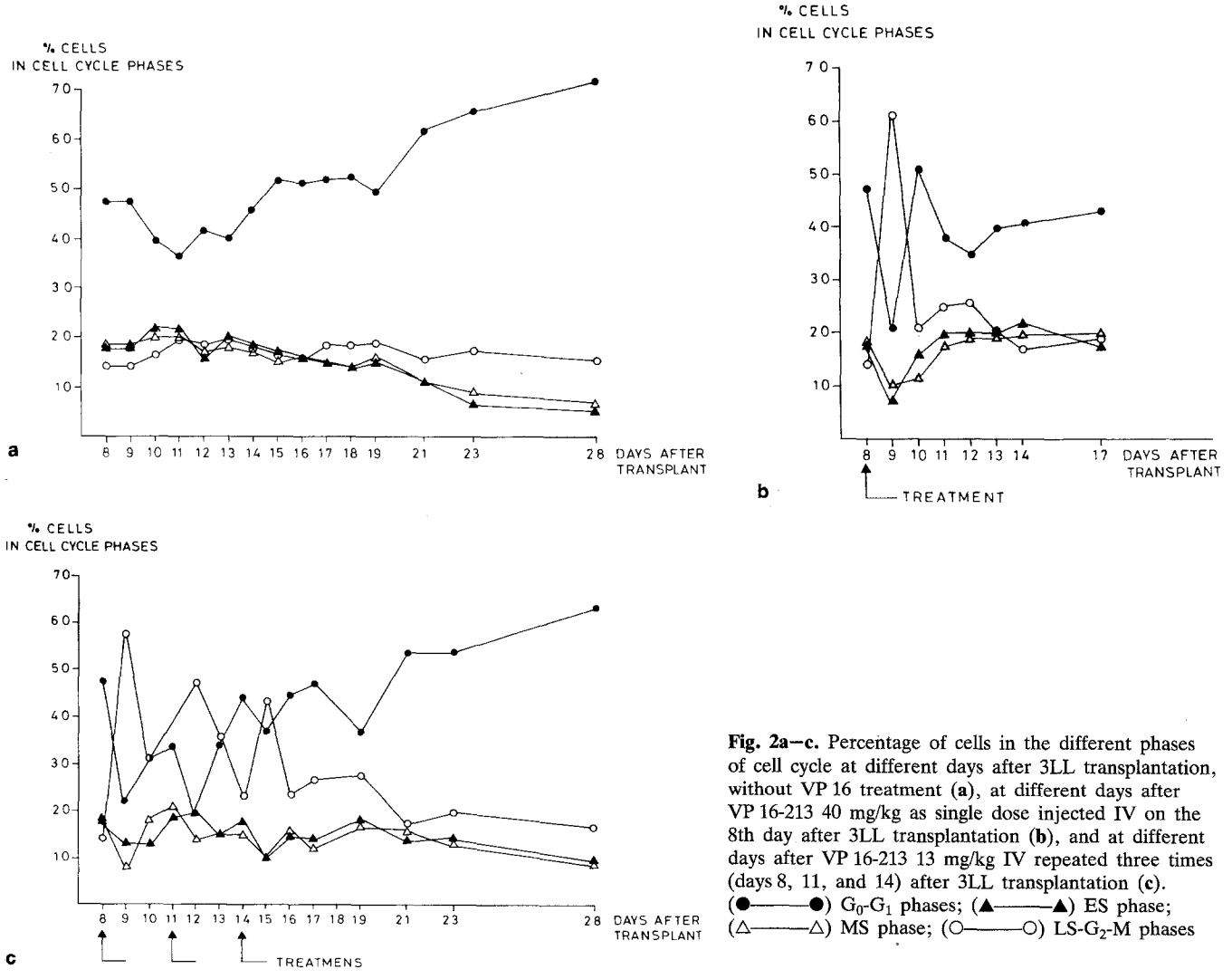

Fig. 2a–c. Percentage of cells in the different phases of cell cycle at different days after 3LL transplantation, without VP 16 treatment (**a**), at different days after VP 16-213 40 mg/kg as single dose injected IV on the 8th day after 3LL transplantation (**b**), and at different days after VP 16-213 13 mg/kg IV repeated three times (days 8, 11, and 14) after 3LL transplantation (**c**). (●——●) G_0-G_1 phases; (▲——▲) ES phase; (△——△) MS phase; (○——○) LS-G_2-M phases

cytofluorographic assay was performed on 20–40 × 10^3 cells. Each count was performed on more than 20,000 cells; the CV of the percentage given is less than 1%.

Results

Figure 1 shows examples of DNA histograms of 3LL cells, obtained by flow cytometry before (Fig. 1a) and 24 after IV VP 16 at doses of 40 mg/kg (Fig. 1b) and 13 mg/kg (Fig. 1c). From left to right, the first peak represents DNA of diploid cells present in the tumor overlapping with normal mouse leucocytes used as standard; the second peak represents the 3LL tumoral cells in G_0-G_1 phase, characteristically tetraploid; the third, less prominent, peak represents the tumor cells in late S (LS)-G_2-M phases of the cell cycle; between the G_1 and LS-G_2-M peaks are cells in S phase.

The DNA patterns of normal cells (2N) present in the tumor appear similar in VP 16-treated and control animals. In contrast, tumor cell distribution differed in VP 16-treated animals, with a lower G_0-G_1 peak and a correspondingly higher LS-G_2-M peak.

Figure 2 shows the percentage of cells in the different phases of the cell cycle at different days after 3LL transplantation without VP 16 treatment (Fig. 2a), after 40 mg/kg as a single IV dose on the 8th day after transplant (schedule A) (Fig. 2b), or after 13 mg/kg IV repeated three times (days 8, 11, and 14) after transplant (schedule B). After either the single dose or any of the repeated doses there was a marked increase in the percentage of cells in LS-G_2-M, with a corresponding decrease in the percentage of cells in G_0-G_1. The accumulation of cells in LS-G_2-M caused by VP 16 became unnoticeable after 24–48 h, when the cytofluorometric pattern became normal. From the plots of Fig. 1, keeping the 2N peak value of diploid cells constant the relative number of total tetraploid cells (G_1 + S + G_2M) appeared to drop after schedule A and was still more reduced after schedule B.

Figure 3 reports the ratios between 4N and 2N cells as a function of the time after transplantation of control and A and B-treated animals. In control animals the ratio increases with time as in treated animals, but in the latter we observe a delayed increase corresponding to the number of treatment given.

This is in line with the finding, illustrated in Fig. 4, that tumor weight inhibition is more pronounced after schedule B than after schedule A. Table 1 shows the antitumor activity and the hematological toxicity of the two schedules of VP 16 in more detail. A better antitumor effect of schedule B is accompanied by lower bone marrow toxicity and better survival.

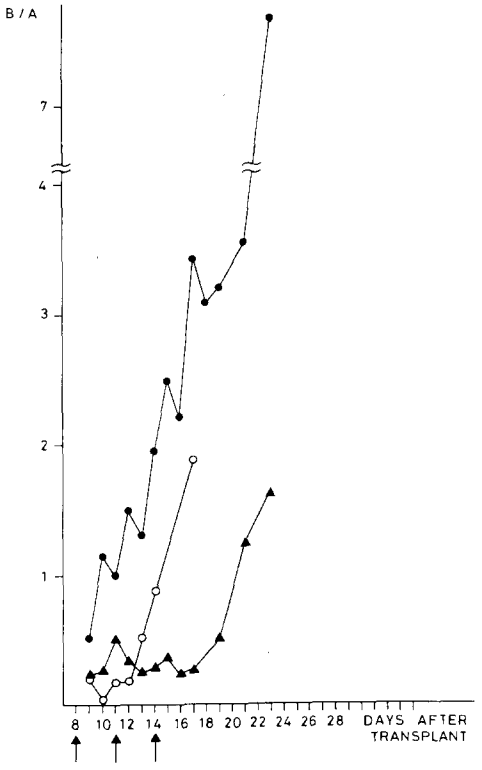

Fig. 3. B/A ratio as a function of the time between tetraploid (4N) cancer cells, B, and diploid normal cells A (2N). The *arrows* indicate the days of IV VP 16 treatment. (●———●) 3LL control; (○———○) 3LL after 40 mg/kg as single dose injected IV on the 8th day after transplant; (▲———▲) 3LL after 13 mg/kg IV repeated three times (days 8, 11, and 14) after transplant

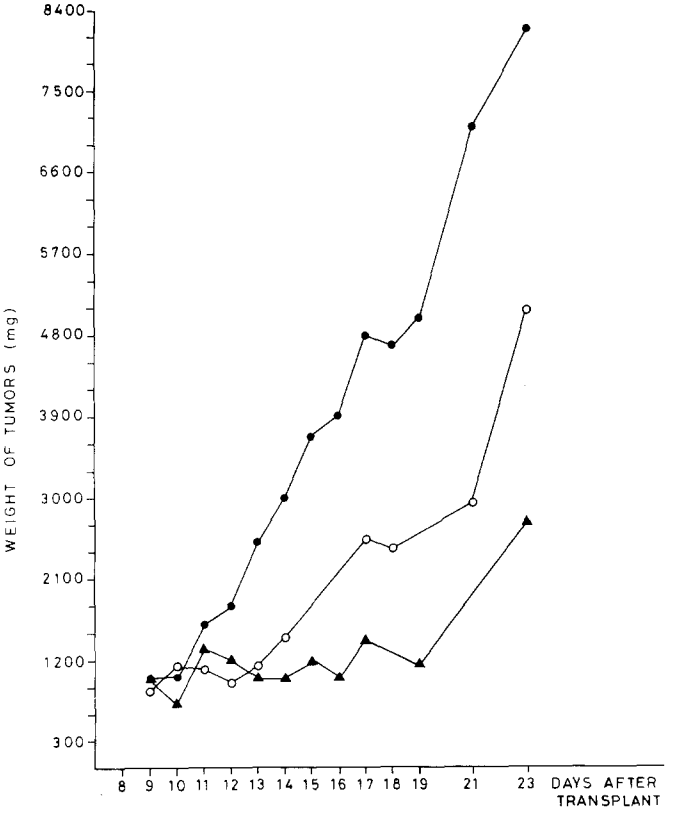

Fig. 4. Tumor weights after transplant. (●———●) 3LL control; (○———○) 3LL after 40 mg/kg as single dose injected IV on the 8th day after transplant; (▲———▲) 3LL after 13 mg/kg IV repeated three times (day 8, 11, and 14) after transplant

Table 1. Antitumoral activity and hematologic toxicities of VP 16 at two different schedules[a] after IV injection in 3LL-bearing mice

	Tumor weight (g)[b]	T/C (%)[d]	Blood cell count (%)[e]		
			WBC	Pt	Ht
Controls	7.53 ± 0.6	–	100	100	100
Schedule A	6.36 ± 0.4	100	33	84	92
Schedule B	3.70 ± 0.3[c]	139	80	100	84

[a] Schedule A = 40 mg/kg on day 8; schedule B = 13 mg/kg on days 8, 11, 14. Each group consisted of 10 mice
[b] Tumor weight on day 25 after tumor transplantation (mean ± SE)
[c] $P < 0.01$ Duncan's test vs controls
[d] T/C = median survival time of treated mice/median survival time of controls × 100
[e] Maximal reduction, % of controls. WBC = white blood cells; Pt = Platelets; Ht = Hematocrit

Discussion

The data presented in this study further corroborates previous findings that VP 16 exerts its antiproliferative effect through a block of cells in a premitotic phase. This cytokinetic effect has been previously reported in a variety of murine and human malignant or normal cells exposed to VP 16 in vitro [4, 7, 11] and recently in vivo on cells from children suffering from acute nonlymphocytic leukemia (ANLL) [13].

Though we have not followed the fate of the cells arrested in SL-G_2-M, considering the data of previous studies on other cell types [4, 11] it is pertinent to hypothesize that they die before entering mitosis. Our data suggest this possibility, as we observed a lower ratio of 4N + 8N/2N cells corresponding to the accumulation of cells in LS-G_2-M phase. In addition, if the observed rapid reversibility of the accumulation of cells in LS-G_2-M was due to progression through the cell cycle, rather than to their death, we should have found larger percentages of cells in G_1 or ES-MS phases subsequent to the block in LS-G_2-M, but we never did.

The cell cycle perturbation after single doses of 13 or 40 mg/kg appears to be similar. Fractioned doses (e.g., 13 mg/kg × 3) have a better chance of achieving better tumor growth control. This advantage of repeated over single doses could in fact be expected on the basis of the demonstrated phase-specificity of VP 16 [4, 7], and is consistent with previous data indicating that the exposure time of 3LL cells to pharmacological VP 16 concentrations is crucial for cytotoxicity [3]. However, in choosing the optimal interval between subsequent VP 16 doses, account must also be taken of the toxic effects on normal tissues, mostly on bone marrow. For example, the best therapeutic index of VP 16 on 3LL was observed when the interval between doses was 3 days, whereas shorter intervals resulted in too high toxicity (data not presented). For a faster-growing tumor, such as L1210, the best schedule seems to be repeated doses every 6 h [14]. This suggests that the interval between doses must be modulated with reference to the kinetics of tumor and normal cells.

This concept must be borne in mind when VP 16 is investigated in human malignancies which present broad kinetic differences. The use of similar VP 16 schedules for rapidly growing tumors such as leukemias or lymphomas and small cell lung cancer (SCLC) or for slow-growing neoplasias such as breast cancer or non-SCLC does not appear rational. Increased knowledge of the kinetic effects of VP 16 on human tumors in vitro is therefore warranted, to provide a basis for more effective dosage schedules.

The present study suggests a way of studying combinations with other phase-specific compounds, which could prove synergistic by acting at different levels of the cell cycle.

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