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In vitro pharmacodynamic evaluation of VP-16-213 and implications for chemotherapy*

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Summary. VP-16-213 (Etoposide) is an active antineoplastic agent which has undergone extensive evaluation of clinical dose escalation. To corroborate a putative dose-response relationship, we studied, in a modified clonogenic assay, various doses and durations of exposure. VP-16-213 at doses of 0.01, 0.05, 0.10, 0.50, 1.0, 5.0, and 10.0 μg/ml, each with exposure durations of 1, 3, 18, and 30 h, was studied in vitro against two human tumor cell lines, MOLT and 9812. The doses and durations of exposure were chosen to approximate some of the pharmacokinetic values achievable in either standard-dose or high-dose clinical studies. The results, summarized as linear regression lines, demonstrate with statistical significance ($p < 0.03$) that there is correlation between dose and cytotoxicity and between dose × duration of exposure (representing the area under the concentration-time curve) and cytotoxicity. Our in vitro data thus support the concept of intensive use of VP-16-213 to maximize antitumor activity. However, how best to accomplish the manipulation of dose and duration of exposure is not yet clear and will be the subject of future clinical investigations.

Introduction

The epipodophyllotoxins are a new and important class of cytotoxic agents demonstrating activity in the treatment of acute leukemia, germ cell carcinoma, lymphoma and small cell carcinoma of the lung [12, 13]. VP-16-213 or etoposide, the most extensively evaluated epipodophyllotoxin, is now commercially available and has a recommended daily dose of approximately 100 mg/m^2 when given for 3–5 days [8, 10]. As a single agent, VP-16-213 at routine dosage produces modest myelosuppression as its predominant toxic effect. With this circumstance in mind, the dose of VP-16-213 has been escalated by accepting substantially more myelosuppression to a maximally tolerated total dose of approximately 2400 mg/m^2; further escalation is contraindicated by mucous membrane toxicity [11, 23]. Pharmacokinetic evaluation of these high-dose studies revealed retention of the standard-dose drug half-life and plasma clearance but markedly higher peak plasma levels and concomitant increase in the area under the concentration-time curve [2, 3, 6, 7, 16]. Although some clinical trials have suggested increased antitumor activity with higher doses, conclusive proof of this is still lacking [11, 21, 22, 23, 24]. Therefore, to help establish a pharmacodynamic rationale for the use of high-dose VP-16-213, we determined in vitro dose-response relationships of VP-16-213 against two human tumor cell lines using various drug concentrations and durations of exposure.

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Materials and methods

Cell lines. MOLT maintained in enriched RPMI with 10% fetal calf serum and 9812 maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum were used as the target cell lines. MOLT was derived from a human T cell lymphoma and 9812 from a human anaplastic bronchogenic carcinoma. Enriched RPMI consisted of RPMI-1640, fetal calf serum, L-glutamine (4 m*M*), penicillin (50 units/ml)/streptomycin (50 μg/ml), sodium pyruvate (1 m*M* GIBCO, Grand Island, NY), and gentamicin (10 μg/ml Schering Corp, Kenilworth, NJ). Our DMEM consisted of basic DMEM fetal calf serum, and penicillin/streptomycin. MOLT was grown as suspension culture in 25-cm^3 tissue culture flasks and 9812 as adherent culture in 75-cm^3 tissue culture flasks (Falcon Plastics, Oxnard, Calif). Cultures were maintained in a 5% CO_2, 37 °C, humidified incubator. Cells were harvested for assay in the log phase of growth.

Assay system. Cytotoxicity was assayed in triplicate using percent reduction of colony growth in treated vs untreated cells in a two-layer agar system as described by Hamburger and Salmon [14]. Briefly, each 35 × 10 mm Petri dish (Falcon Plastics, Oxnard, Calif) contained a 1-ml feeder layer overlaid by a 1-ml layer containing the cells. The feeder layer was prepared from the stock culture media supplemented with 0.5% tryptic soy broth (DIFCO, Detroit Mich), DEAE-Dextran (0.24 mg/ml; Pharmacia, Uppsala, Sweden), and L-asparagine (64 μg/ml; GIBCO, Grand Island, NY) mixed with bacto agar (DIFCO, Detroit, Mich) yielding a final agar concentration of 0.5%. The top layer was prepared from the cells in their appropriate media mixed with agar to yield a final agar concentration of 0.3%. Experiments were reviewed microscopically within 24 h of plating to assure that single-cell suspensions were used. Dishes containing clumps of cells were discarded. Colonies (defined as greater than 30 cells)

were counted 14 days after plating using an inverted microscope ($\times 40$) by one individual blinded as to which dish was being counted.

Drug preparation. VP-16-213 (etoposide, Bristol Laboratories, Syracuse, NY) was diluted with normal saline just prior to use. As an additional control, the solvent for the drug was prepared from the manufacturer's instructions: 400 mg Tween-80, 3.25 g polyethylenglycol, 10 mg anhydrous citric acid (Sigma Chemicals, St Louis Mo), 150 mg benzyl alcohol, and absolute alcohol to bring the final volume to 5 ml. Dilutions of the solvent corresponding to the concentrations found in the appropriate drug dilution were used as controls for evaluation of cytotoxicity.

Drug exposure. Cell lines were collected, counted, and viability determined by trypan blue exclusion. Next, 1.15 ml fresh media, 0.20 ml MOLT cells (0.75×10^4 ml) or 9812 cells (2.25×10^4/ml), and 0.15 ml drug (or normal saline control) were mixed in a 15-ml conical polystyrene tube (Falcon Plastics, Oxnard, Calif) and incubated for 1, 3, 18, or 30 h. After completion of incubation, the tube was centrifuged at 1500 rpm for 5 min and the supernatant was removed and replaced with fresh media. Washing was repeated twice more and the final cell pellet was resuspended with 2.7 ml media.

Statistical methods. Results were analyzed using the CLINFO data system. Linear regression was calculated by the least squares method. Curves were forcefitted through the origin. For regression computations, values of dose or dose $\times$ duration were not included in the calculations once 100% cytotoxicity was achieved.

Results

Solvent cytotoxicity

Solvent alone at concentrations equivalent to that present in experiments with 1 μg/ml VP-16-213 caused less than 10% cytotoxicity with exposure from 1 to 30 h. Higher solvent concentrations for short exposure durations (1 and 3 h) also caused no substantial cytotoxicity. Results are therefore not adjusted for solvent cytotoxicity.

VP-16-213 cytotoxicity

Cytotoxicity was studied at seven doses, 0.01, 0.05, 0.10, 0.50, 1.0, 5.0, and 10.0 μg/ml, each at four durations of exposure, 1, 3, 18, and 30 h. Each experiment (performed in triplicate) was repeated and the results are summarized in graphic form in Fig. 1 and Fig. 2 for MOLT cells and 9812 cells respectively. The lines drawn are linear regression analyses forcefitted through the origin. As shown, with increasing dose of drug there was increasing cell cytotoxicity. Additionally, for each dose, there was increasing cytotoxicity with increasing duration of exposure. For regression calculations, once maximal (100%) cytotoxicity was observed, higher doses were not included in the regression computations. Tables 1 and 2 summarize the regression lines: r values ranged from 0.70 to 0.89. Also shown is the lowest dose producing maximal cytotoxicity. As the duration of exposure increases, the dose required to produce maximal cytotoxicity decreases. To evaluate the influence of the area under the concentration-time curve, cytotoxicity was compared with dose $\times$ duration of exposure. Graphic representations of linear regression lines are shown in Figs. 3 and 4 for MOLT cells and 9812 cells respectively. All possible dose $\times$ duration values were calculated and ranged from 0 (control) to 300 μg·hr/ml. As demonstrated in these plots, maximal cytotoxicity was observed at dose $\times$ duration values of 8–10 μg·hr/ml. As for the previous plots, lines were forced through the origin. The r and p were 0.83 and <0.001 for MOLT cells and 0.80 and <0.001 for 9812 cells.

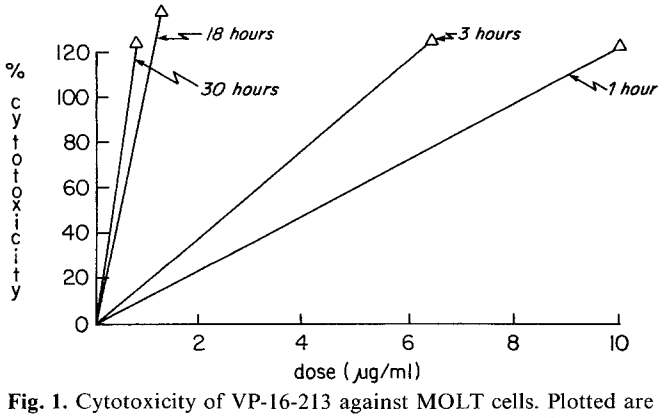

Fig. 1. Cytotoxicity of VP-16-213 against MOLT cells. Plotted are the dose-response relationships at constant durations of exposure. Curves are linear regressions forced through the origin

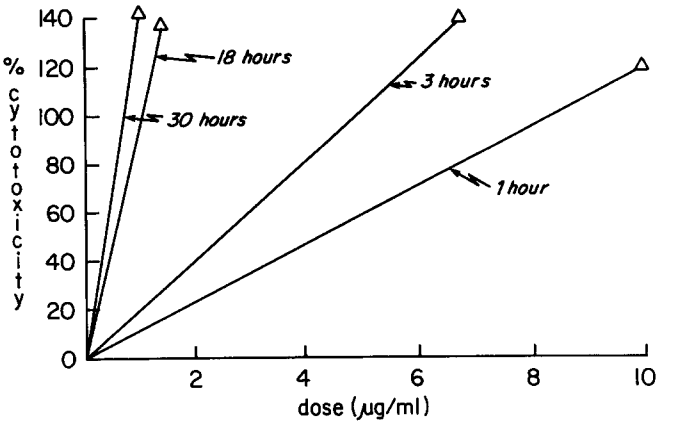

Fig. 2. Cytotoxicity of VP-16-213 against 9812 cells. Plotted are the dose-response relationships at constant duration of exposure. Curves are linear regressions forced through the origin

Table 1. VP-16-213 cytotoxicity against MOLT cells: evaluation of the effect of dose

Duration of exposure	r	p	Maximal cytotoxicity
1 h	0.86	<0.001	10 μg/ml
3 h	0.71	0.005	5 μg/ml
18 h	0.88	<0.001	1 μg/ml
30 h	0.85	0.002	0.5 μg/ml

Table 2. VP-16-213 cytotoxicity against 9812 cells: evaluation of the effect of dose

Duration of exposure	r	p	Maximal cytotoxicity
1 h	0.89	<0.001	10 μg/ml
3 h	0.79	<0.001	5 μg/ml
18 h	0.85	<0.001	1 μg/ml
30 h	0.70	0.027	0.5 μg/ml

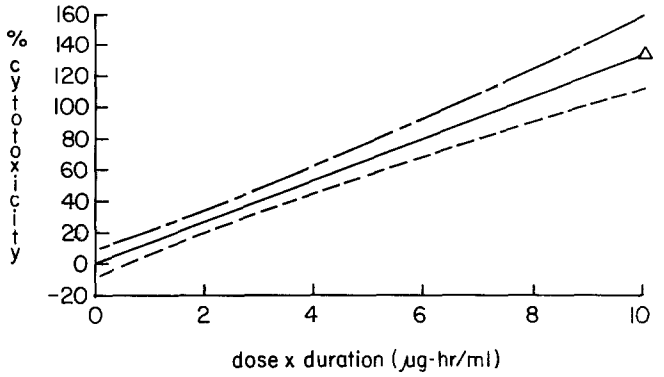

Fig. 3. Cytotoxicity of VP-16-213 against MOLT cells. Plotted is the value of the dose × duration of exposure (representative of the area under the concentration-time curve) vs cytotoxicity. The lines are linear regressions force-fitted through the origin with 95% confidence limits

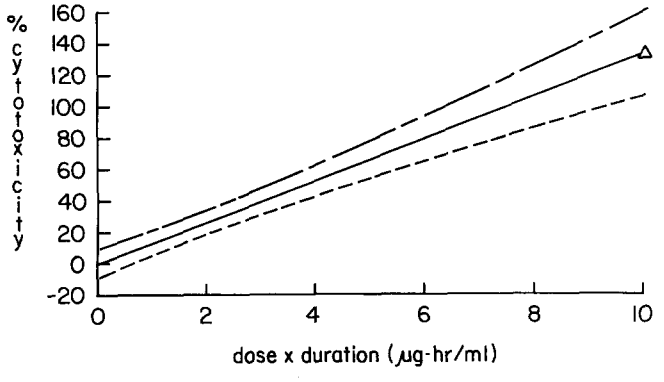

Fig. 4. Cytotoxicity of VP-16-213 against 9812 cells. Plotted is the value of the dose × duration of exposure (representative of the area under the concentration-time curve) vs cytotoxicity. The lines are linear regressions force-fitted through the origin with 95% confidence limits

Discussion

VP-16-213 (etoposide) is now a widely used antineoplastic agent which has been extensively dose-escalated. This study evaluated a wide variety of drug doses against an in vitro model to corroborate a dose-response relationship.

We modified the standard tumor stem cell assay to incorporate not only dose as a variable, but also a wide range of durations of drug exposure. We selected values of these parameters to approximate in vitro the in vivo pharmacokinetics in man. Noting that the terminal half-life of VP-16-213 is approximately 5–10 h, and with standard-dose to high-dose peak plasma levels ranging from 10 to 100 μg/ml, we selected suitable doses and duration of exposure. For example, with standard-dose administration, VP-16-213 plasma levels are generally <1 μg/ml within 24 h, but with high-dose infusions are >1 μg/ml. However, it would be unwise to suggest anything more than a qualitative comparison between the in vitro model and the in vivo situation, since the model cannot capture dynamic in vivo circumstances [18–20].

Our results demonstrate that there is both a dose-response relationship and a dose × duration relationship between VP-16-213 and cytotoxicity. Linear regression curves drawn through the origin demonstrate the significance of these relationships. Dose-response relationships have also been reported previously, although in more restricted dose ranges [4, 5]. Therefore, our in vitro data would support the concept of high-dose therapy and further suggest that methods to increase the area under the concentration-time curve be sought in order to maximize antitumor activity. However, maximizing the area under the concentration-time curve may also increase toxicity, thus negating any improvement in therapeutic ratio. Additionally, our experimental design is unable to determine whether there is a cytotoxic difference between short exposures to high concentrations and prolonged exposures to lower drug concentrations. Experiments designed to answer this question are now in progress.

Besides administration of high doses, another method of sustaining plasma levels is the use of continuous infusion. To date, three studies have been reported using this technique [1, 9, 15]. Doses were from 60–150 mg/m^2/day administered for 5 days. For the larger doses, maintenance of plasma levels over 1 μg/ml would be anticipated. Indeed, in these studies, evidence of both tumor response and severe toxicity (myelosuppression) was observed even at the lower concentrations.

How best to administer VP-16-213 in clinical situations remains a topic for speculation. Although there are now clinical and laboratory data to warrant intensification of VP-16-213 administration, how to manipulate dose and duration of exposure is not yet clear. Clinical studies to evaluate these parameters are necessary. A recent preliminary report compared two methods of administration of VP-16-213, with both schedules designed to yield similar areas under the concentration-time curve [17]. VP-16-213 was administered either as a 24-h infusion of 500 mg/m^2 or as five daily 2-h infusions of 100 mg/m^2. Twenty patients received the single infusion and 18 patients received multiple infusions. Interestingly, the response rate was significantly greater in the group receiving multiple infusions. In that study, although the pharmacokinetics were similar, there appeared to be improved cytotoxicity with longer durations of exposure. Early phase I evaluations of VP-16-213 had also suggested the superiority of multiple daily infusions to single-dose administration. With the availability of pharmacokinetic data, specific dosing intervals can now be chosen to maintain and maximize the area under the concentration-time curve. In this context, our next clinical study will evaluate the antitumor activity of high-dose VP-16-213 administered for 3 alternate days as opposed to the previous schedule of 3 consecutive days in order to increase the area under the concentration-time curve without modifying peak plasma levels.

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