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In Vitro Cytotoxicity of VP 16 on Primary Tumor and Metastasis of Lewis Lung Carcinoma*

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Abstract—*VP 16 activity was investigated on cells derived from primary tumor or metastases of Lewis lung carcinoma grown* in vitro. *After* 24 hr *exposure to VP 16 we found a marginal effect at* 0.1 μg/ml, *but at* 1 *and* 10 μg/ml [^{3}H]-*TdR uptake was* 37 *and* 85% *less than controls for primary tumor and* 43 *and* 83% *for metastases; cell numbers dropped to* 41 *and* 76% *of controls for primary tumor and* 30 *and* 68% *for metastases. After* 72 hr *exposure VP 16 had a similar effect on cells of primary tumor or metastases, but a cytotoxic effect was already evident at* 0.01 μg/ml *and increased proportionally to the concentration reaching virtual complete cell killing at* 1 μg/ml. *This study suggests that the much greater* in vivo *sensitivity to VP 16 of metastases than primary tumor of Lewis lung carcinoma probably arises less from an intrinsically greater susceptibility of metastatic cells than from pharmacokinetic factors.*

INTRODUCTION

VP 16 213 (VP 16) or 4′ - demethylepipodophyllotoxin - 5 - (4,6 - *O* -) - ethylidene - β - D - glycopyranoside (NSC 141540) is a semisynthetic podophyllotoxin derivative reported to be active in several experimental and human malignancies [1–4]. Its mechanism of action has been only partially elucidated: in contrast with podophyllotoxin, VP 16, at pharmacologically reasonable concentrations, does not appear to be a spindle poison but acts in the G2 and S phase of the cell cycle [4].

Both VP 16 and its congener VM 26 reportedly possess a marked antimetastatic effect on Lewis lung carcinoma (3LL) [5] without significant cytotoxicity against the primary tumor, but the reason for such a phenomenon has not yet been clarified. One possibility is that these drugs achieve higher concentrations in metastases which are better vascularized than the primary tumor. Another hypothesis is that metastatic cells are intrinsically more sensitive to podophyllotoxin derivatives.

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The latter hypothesis was tested in this study in which the susceptibility of 3LL cells of primaries and secondaries growing *in vitro* was explored at different concentrations and time exposures of VP 16.

MATERIALS AND METHODS

Two methods were used to obtain cell cultures from primary tumors and metastases. Tumor fragments were removed aseptically from C57B1/6 mice 18 days after intramuscular transplant of 2×10^5 viable cells of a syngeneic Lewis lung carcinoma (3LL) (maintained by intramuscular passages every 2 weeks in the same strain) and washed in a Petri dish with phosphate-buffered saline (PBS) supplemented with antibiotics (penicillin–streptomycin solution, 10,000 units/ml).

The tumor fragments were minced with fine sterile scissors to separate the vegetant from the necrotic part. Trypsin 1:250 (Difco certified), 40 ml, 0.25% in PBS was used to obtain a cell suspension after 30 min of incubation at 37°C in continuous shaking. After enzymatic disgregation 20–30 ml of fetal calf serum (Gibco) were added to stop trypsin digestion; the cell suspension was filtered through 7–8 layers of sterile gauze, counted by

the dye exclusion method with trypan blue, and 1×10^5 cells per ml were seeded in growth medium in 24-well tissue culture cluster dishes (Nunc) and incubated at 37°C. Growth medium was 199 (Gibco BioCult, Glasgow, Scotland) supplemented with 20% fetal calf serum, 2 mM glutamine and containing 4 x extra amino acids and 4 x extra vitamin solution, buffered with 20 mM Hepes (2 - 4 - 2 - hydroxy - ethyl - piperazinyl - 1 - ethane - sulfonic acid, Merck) [6]. To obtain cells from metastases, mice were killed 25–30 days after the inoculum. In this case trypsin 0.25% and DNase 2.5 mg/ml (Deoxyribonuclease I, Type 1, Sigma Chemical Company, St. Louis) were used in Hank's Balanced salt solution (BBL, Maryland), respectively 20 ml and 0.8 ml, to obtain cell suspensions [7]. The procedure, inoculum and growth medium were the same as for the primary tumor. After 24 hr incubation the culture medium was replaced with fresh medium and after 48 hr it was with a medium containing different concentrations of the drug.

The growth rates of cells of primary tumor and metastases were very similar throughout the duration of our experiments.

VP 16 was dissolved in media plus Tween 80 (Polyoxethylensorbitanmonooleate) (Merck) to obtain a 0.001% concentration of Tween 80, which is not toxic to cells of primary tumor or metastases *in vitro*.

In these experiments the exposure time ranged from 24 to 72 hr. At the end of treatment or recovery period with drug-free medium the wells were emptied, washed once with PBS and the effect of the treatment was evaluated. The stability of VP 16 at 37°C in human plasma has already been reported [8] and was confirmed by HPLC assay in 199 Medium in which less than 10% of degradation was observed after 72 hr.

The cytotoxicity of VP 16 was assessed 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 6 hr of treatment or recovery time; and (b) as a reduction in the cell count made using a Coulter Counter Model ZB (Counter Electronics Ltd., Northwell Drive, U.K.). Dunnett's test was employed for statistical analysis of results, using a total of 10 samples for each exposure time and each concentration.

RESULTS

Figure 1A and B shows the effect of VP 16 at concentrations of 0.1, 1 and 10 μg/ml on 3LL primary tumor incubated with the drug for 24 hr. A significant decrease in cell number was observed at concentrations of 1 and 10 μg/ml; the VP 16 concentration–cytotoxic effect relationship appears to be linear ($P < 0.01$).

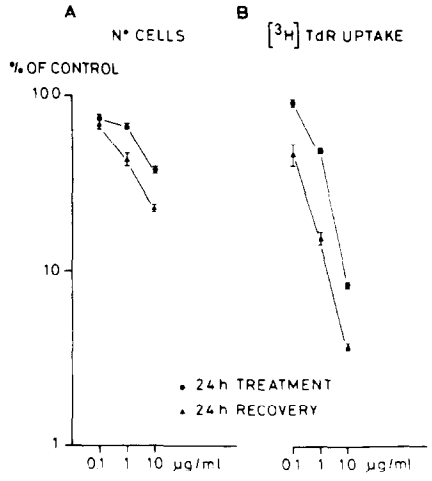

Fig. 1. Cell cytotoxicity and inhibition of [^{3}H]-*TdR uptake caused by VP* 16 *at concentrations of* 0.1, 1 *and* 10 μg/ml *on cells derived from 3LL primary tumor grown* in vitro. ●——● *After* 24 hr *exposure;* ▲——▲ *after* 24 hr *recovery with drug-free medium.*

The results were essentially similar when cell count or [^{3}H]-TdR uptake was used to assess the drug's cytotoxic effect, which was not reversed after 24 hr of recovery during which cells were incubated with drug-free medium. A second experiment was made to compare VP 16's cytotoxic effect on primary tumor and lung metastatic cells, both exposed to the drug for 24 hr. [^{3}H]-TdR uptake by primary and secondary cells was reduced to a very similar extent; there was no effect at 0.01 and 0.1 μg/ml. At concentrations of 1 and 10 μg/ml there was a reduction of TdR uptake proportional to the concentration (see Fig. 2).

The results of this experiment were also evaluated by morphological examination of the cells before and after treatment; severe cell damage with areas of cell destruction were observed after VP 16 at 10 μg/ml and, to a lesser extent, at 1 μg/ml. Only a decrease of cell number without any apparent morphological difference from controls was seen at 0.1 μg/ml and no effect at all was visible at 0.01 μg/ml. Subsequent experiments were performed to assess the importance of exposure time for the effect of VP 16 on primary and metastatic cells. Figure 3 reports the cytotoxic effect after 24 and 48 hr of treatment, and on cells from primary tumor after 72 hr too. The cytotoxic effect appears to increase proportionally to the exposure time. This was confirmed when 3LL

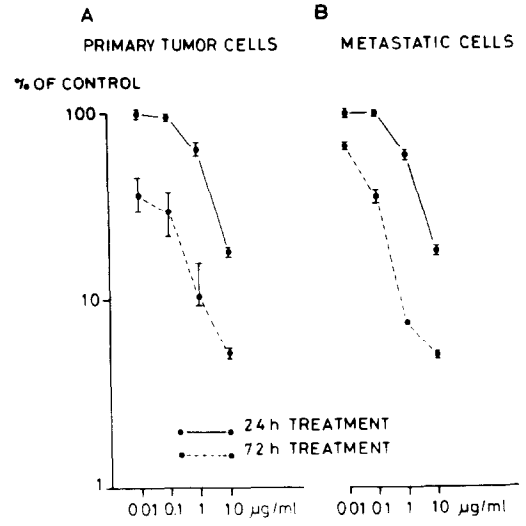

Fig. 2. Comparison of the inhibition of [^{3}H]-*TdR uptake of cells derived from primary tumor or metastases of 3LL grown* in vitro, *both exposed to VP* 16 *at concentrations from* 0.01 *to* 10 μg/m. ●——● *After* 24 hr *exposure*; ●---● *after* 72 hr *exposure.*

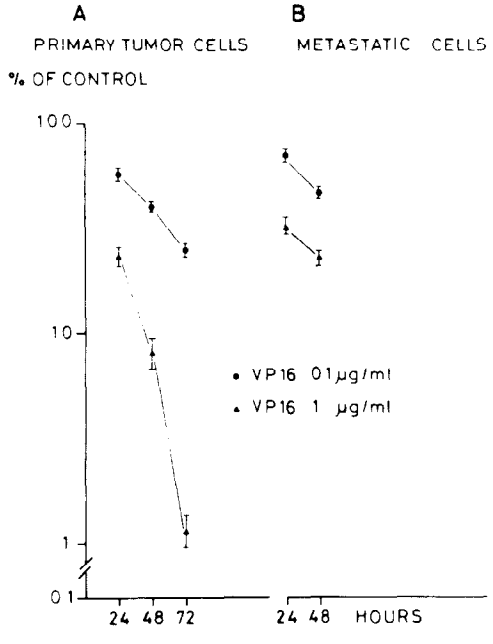

Fig. 3. Comparison of the cytotoxicity of VP 16 *on cells derived from primary tumor or metastases of 3LL grown* in vitro *exposed to VP* 16. *Cells of primary tumor were exposed for* 24, 48 *and* 72 hr, *cells from metastases for* 24 *and* 48 hr. ●——● *VP* 16 *concentration*: 0.1 μg/ml; ▲——▲ *VP* 16 *concentration*: 1 μg/ml.

cells from primary and secondary neoplasms were incubated for 72 hr with VP 16 at concentrations of 0.01, 0.1, 1 and 10 μg/ml, and [^{3}H]-TdR uptake was measured (see Fig. 2).

No viable cellular elements of primary tumor or metastasis were found after treatment with VP 16 at 1 or 10 μg/ml for 72 hr; a marked effect was observable after 0.1 μg/ml but 0.01 μg/ml did not affect the morphology of cells compared to controls in spite of a slight, but significant ($P < 0.05$) reduction of TdR incorporation.

DISCUSSION

Cells derived from primary tumor or metastases of 3LL appeared to be similarly affected by VP 16 in our experimental conditions. As far as we know there are no other studies on cell sensitivity of 3LL to VP 16. However, when other cell types were exposed to increasing VP 16 concentrations a similar exponential survival curve was described [9].

To make any correct comparison between the *in vitro* and *in vivo* cytotoxicity of an antineoplastic compound it is necessary *in vitro* to use a range of drug concentrations which are realistically achieved *in vivo* in tumor and metastatic cells. Pharmacokinetic data are, however, rarely available for the tumor model which is under investigation *in vitro*. For this reason our group previously investigated the distribution of VP 16 in 3LL-bearing animals [10] using a specific analytical assay [8]. The concentration × time ($C \times T$) values for VP 16 in primary tumor or metastases after giving 40 mg/kg i.v., a dose which has a striking anti-metastatic effect with only a weak inhibitory effect on primary tumor [5], were approximately 17 and 63 μg/g × hr respectively for primary tumor and metastases. VP 16 at 0.1 μg/ml for 24 hr, which was not effective on 3LL grown *in vitro*, corresponded to a $C \times T$ value of 2.4 μg/g × hr, thus much less than the $C \times T$ values found *in vivo*. The concentration of 1 μg/ml for 24 hr, which was, however, active *in vitro*, corresponded to a $C \times T$ value of 24 μg/ml × hr, only slightly higher than the value found *in vivo* in the primary tumor and 2.6 times less than that in metastases.

From these considerations the correlation between the *in vitro* and *in vivo* cytotoxicity of VP 16 appears to be good in this model when *in vitro* drug concentrations similar to those achieved *in vivo* are investigated.

The fact that metastatic cells appear to be as sensitive as primary tumor cells suggests that the higher susceptibility to VP 16 of metastases observed *in vivo* [5] is likely to be due more to preferential distribution of VP 16 to metastases than to a higher intrinsic sensitivity. Another possible explanation is the fast growth rate of metastatic cells of 3LL [11] with a higher percentage of cells in cycle and thus likely to be more susceptible to VP 16 action; this hypothesis is currently under investigation.

As was to be expected on the basis of the mode of action of VP 16, 3LL cell survival and

[^{3}H]-TdR uptake appear to be greatly influenced by the exposure time of VP 16: drug concentrations of 0.01 and 0.1 μg/ml for 72 hr induced significant cell kill and reduction of [^{3}H]-TdR uptake, whereas no such effect was observed after 24 hr exposure unless the concentration was raised at least to 1 μg/ml.

The importance of the contact time in determining the effect of VP 16, which has already been described on other cell types [6], could have some implications for better use of this drug. There is, in fact, an indication that the effect on VP 16 on 3LL can be dramatically improved without increasing bone marrow toxicity if the same dose is fractionated in a 3 times daily schedule (data not published). This may offer the rationale for clinical studies using fractionated doses or continuous infusion in order to expose the malignant cells for a longer time.

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