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Exposure and Schedule Dependency of Etoposide in Neuroblastoma and Leukaemia Cells *In Vitro*

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Using an *in vitro* clonogenic assay system, we examined the relationship between concentration, duration and schedule of exposure to etoposide and cytotoxicity in three cell lines. Two cell lines (SK-N-SH and IMR 32) were derived from human neuroblastomas, and one (L1210) was the original murine leukaemia cell line used to define schedule dependency of etoposide *in vivo*. Cytotoxicity was found to be determined by the product of concentration and duration of exposure over a 120-fold range of durations and a 100-fold range of concentrations. No difference in cytotoxicity was seen following equivalent exposure either continuously or in two separate intervals. In one cell line, exposure to etoposide when at confluence led to a highly resistant subpopulation comprising 10–15% of the entire cell number. This did not seem to be associated with any difference in the rate of etoposide efflux from cells preloaded with ^{3}H-etoposide. We conclude that etoposide does not show schedule dependency *in vitro*, but cytotoxicity is related to total exposure to etoposide. The schedule dependency seen *in vivo* may possibly arise from host pharmacokinetic factors.

Key words: etoposide, neuroblastoma, L1210, cell cycle, schedule dependency
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INTRODUCTION

ETOPOSIDE IS one of the most active and widely used agents in paediatric oncology. Single agent activity has been shown against leukaemia, lymphoma, neuroblastoma, soft tissue sarcoma, germ cell tumour and Wilms' tumour, and in the United Kingdom over 70% of children with malignant disease will receive etoposide at some time.

There is considerable evidence for marked dose and schedule dependency of etoposide activity *in vivo*. Early preclinical studies in the murine L1210 leukaemia model showed that administration every 3 h for 2 days at 5 day intervals was associated with a high rate of cure [1]. More recently, Slevin and associates have reported a trial of etoposide given to patients with small cell lung cancer as either a single infusion of 500 mg/m^2 over 24 h, or 5 daily infusions of 100 mg/m^2 over 2 h [2]. Response rates differed markedly between the two groups, being 10% with a single dose and 87% with divided doses. On the basis of these findings, numerous other studies have been performed investigating the effect of more prolonged schedules of administration both intravenously and orally, and high response rates have been reported [3]. The current UKCCSG NAG 8 protocol involves etoposide given orally over 21 days, and is based on high reported efficacy in adult patients treated for small cell lung cancer with similar prolonged schedules (references reviewed in [4]).

In addition to schedule, dose intensity, or the amount of drug delivered to the patient in a given unit of time has been correlated with clinical efficacy of etoposide [5], and this has led many workers to pursue high dose, rapid schedule protocols, with promising results. The importance of dose intensity of the podophyllotoxins in neuroblastoma was demonstrated in a retrospective study by Cheung and associates [6] in which the dose intensity of teniposide was shown to correlate with median survival and median progression free survival.

A further important factor in etoposide therapy is pharmacokinetic variability. Conventional dosing according to body surface area or body weight has been shown to produce at least a 4-fold variation in patient exposure to etoposide [7], and studies in adult patients have demonstrated a correlation between pharmacokinetic parameters, usually either steady state concentration or area under the concentration–time curve, and pharmacodynamic parameters, either haematological toxicity or tumour response [8–11]. In children, no equivalent study with etoposide has been published, but Rodman and colleagues performed a study of children with neuroblastoma, leukaemia and lymphoma, treated with teniposide as a continuous infusion over 72 h [12]. A highly significant difference was seen between the steady state concentration attained in patients who responded, and those who did not respond, and a threshold concentration of 12 μg/ml was identified, below which the likelihood of response was significantly reduced.

Etoposide is a semi-synthetic derivative of podophyllotoxin, which acts by inhibition of one of the most abundant nuclear proteins, DNA topoisomerase II. It is cell cycle specific, and leads to an accumulation of cells in the G2/M phase of the cell cycle. *In vitro* cytotoxicity studies have also demonstrated the importance of both concentration and duration of exposure to

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etoposide, but the relative importance of these, and of the schedule of administration, has not been fully examined [13–17]. It seems likely, therefore, that dose, schedule and concentration of drug attained may be important determinants of the activity of etoposide in neuroblastoma. The aim of this study was to examine the relative importance of these parameters using a simple *in vitro* system.

MATERIALS AND METHODS

Stability of etoposide in growth medium

Etoposide was dissolved initially in methanol at 1 mg/ml, diluted in growth medium to give a final concentration of 25 μg/ml, and incubated at 37°C in 5% CO_2 for intervals of up to 5 days. Aliquots were removed at intervals and stored at −20°C until analysis, which was by HPLC according to a previously published method [18].

Cytotoxicity of etoposide

Cytotoxicity studies were performed using three established cell lines. Two of these, SK-N-SH and IMR 32, were derived from human neuroblastoma tissue. The murine leukaemia cell line, L1210, was included because this was the original line used to define schedule dependency *in vivo*. All cells were originally obtained from the ATCC (Rockville, Maryland, U.S.A.).

Exponential neuroblastoma cells were plated in 25 cm^2 flasks in growth medium (Eagle's Minimal Essential Medium containing 10% fetal calf serum (FCS) with added glutamine, pyruvate and non-essential amino acids; Life Technologies, Paisley, Scotland) and allowed to adhere. After 24 h, etoposide was added to the desired final concentration, and left for the required interval. For exposures in excess of 24 h, medium and etoposide were replaced every 24 h. After the required exposure time, cells were washed twice in Dulbecco's phosphate buffered saline solution A (DulA), detached from culture flasks by trypsinisation, syringed gently to give a single cell suspension, and diluted to give the required number of cells to be cloned. Cytotoxicity was determined using a soft agar clonogenic assay system similar to that used by Whelan and Hill [19], and colonies were grown in tubes. For counting, tubes were everted into Petri dishes, colonies stained with MTT, and counted by eye under 3x magnification.

L1210 cells grow in suspension, and hence after exposure to etoposide, cells were centrifuged (450 ***g*** for 5 min) and resuspended in fresh medium (Roswell Park Memorial Institute 1640 medium with 10% fetal calf serum and added glutamate) before assay of clonogenicity. Control cloning efficiencies for SK-N-SH, IMR 32 and L1210 cells were (mean ± SD) 18 ± 8%, 25 ± 9% and 73 ± 18%, respectively. The cytotoxic effect of etoposide following exposure of cells to a variety of concentrations, durations and schedules was investigated in both exponentially growing and confluent cells. In each case, clonogenicity was measured as a percentage of the appropriate control clonogenicity.

The majority of studies were performed using SK-N-SH cells. Repeat assays were performed for incubations between 2 h and 5 days, and for exposures which were either continuous or intermittent. Intermittent exposures were for 1, 2 and 24 h with a 24 h interval in drug free medium between exposure. Control cells were near-confluent after 5 days incubation, and IMR 32 cells in particular tended to become detached with changes of medium. It was not possible to examine longer durations of exposure without subculturing cells within an experiment.

VP16 efflux studies

Efflux of etoposide from preloaded cells was measured as follows. 3×10^7 cells in exponential or confluent phases of growth were harvested from dishes with DulA/EDTA, and syringed gently to obtain a single cell suspension. This suspension was centrifuged (400 ***g***, 5 min) and cells resuspended in 3 ml growth medium containing 60 μl ^{3}H-etoposide (382 mCi/mmol, 770 μg/ml, Moravek Ltd, Brea, California, U.S.A.) at 25°C. Cells were agitated gently for 80 min before washing three times with ice cold growth medium, and resuspension in 3 ml fresh growth medium at 25°C. Aliquots of 100 μl were removed at intervals and layered over oil (100 μl, specific gravity 1.028) which was itself layered over 50 μl 3 M potassium hydroxide in narrow Eppendorff tubes. Samples were centrifuged (8600 ***g***, 1 min), causing the cells to pass through the oil layer into the potassium hydroxide layer, where they were lysed.

RESULTS

Stability of etoposide in growth medium

Spontaneous degradation of etoposide was seen after even short periods of incubation at 37°C. The decrease in etoposide concentration approximated to a simple monoexponential decay, with a half life of approximately 1 day (Figure 1). Coincident with this, a second peak was identified by HPLC, rising to a steady state concentration after 36 h. These data are consistent with spontaneous breakdown to a more stable product, believed to be the *cis*-isomer, which has no cytotoxic activity [20]. Calculation of total exposure to etoposide with prolonged incubation could therefore be made by integration of the area under the concentration–time curve, using a simple exponential equation.

$$\mathrm{AUC} = \int_0^t C_{\max} e^{-kt} = \frac{C_{\max}}{k} e^{-kt},$$

where C_{max} = initial concentration, t = incubation period, and k = decay constant for etoposide breakdown *in vitro*.

Cytotoxicity of etoposide in vitro

In each case, for a given duration of exposure, a threshold concentration could be identified, below which cytotoxicity

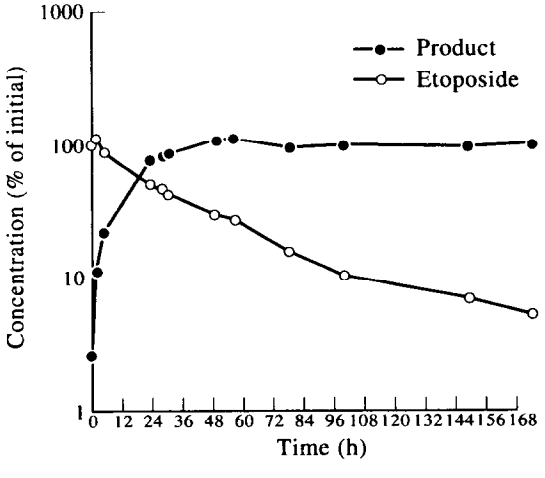

Figure 1. Stability of etoposide in growth medium at 37°C.

was not seen. A characteristic sigmoidal relationship between etoposide concentration and cytotoxicity was seen. IC50 values were lower following prolonged exposure than after short exposure (Figure 2).

In all of the three cell lines analysed, equal cytotoxicity was produced by equivalent total exposures, regardless of total duration or schedule, even when the surviving fraction was less than 0.1% of control values (Figure 3). Repeated assay with exposures of 50 μg/ml for 4 h or 10.8 μg/ml for 24 h, treatments which give identical exposures to etoposide (189 μg/ml × h), produced equivalent cytotoxicity in IMR 32 cells.

Similarly, no difference in cytotoxicity was seen following equivalent total exposure to etoposide, regardless of whether continuous or intermittent treatment was used. Exposure for intervals of 1, 2 and 24 h on two occasions, separated by a 24 h drug free interval, was compared with similar equivalent continuous exposure over 2, 4 and 48 h, respectively. The surviving fraction of cells was identical for equivalent total exposure (Table 1).

In a further series of experiments, IMR 32 cells were allowed to reach confluence, with daily changes of growth medium, and exposed to etoposide for 24 h. AUC50 values were identical to those seen with exponential cells (1.7 μg/ml × h), but between 10 and 15% of cells were consistently found to be resistant to even very high exposures to etoposide (up to 86 μg/ml × h, Figure 4). Subsequently, these cells were subcultured for 9 passages in drug free medium, and retained a resistant phenotype (Figure 5).

VP16 efflux studies

Results of efflux experiments are shown in Figure 6. No difference in rates of efflux of etoposide from preloaded cells was seen in SK-N-SH cells, and more rapid efflux was seen from exponentially growing IMR 32 cells than from confluent cells. After 45 min, however, equivalent amounts of etoposide had been cleared from exponential and confluent cells.

DISCUSSION

Given the known cell cycle specificity of etoposide *in vitro* [15], and schedule dependency *in vivo* [3, 4], it is perhaps surprising that no optimum schedule could be identified in our study. Schedule dependency may arise *in vivo* as a result of either tumour or host-related characteristics: administration of divided or continuous low concentrations of drug may lead to recruitment of cells into sensitive phases of the cell cycle, and

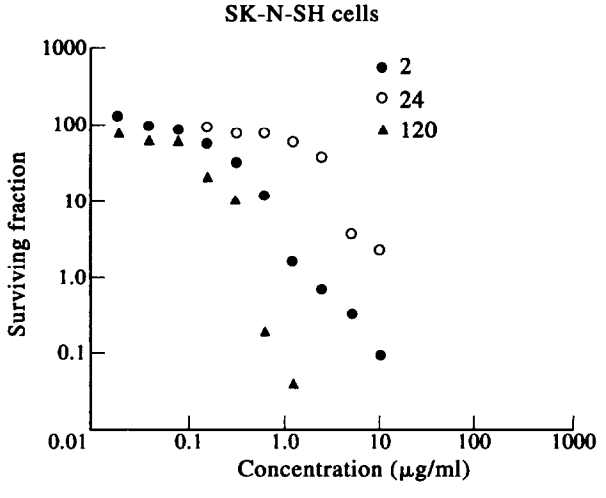

Figure 2. Clonogenicity of SK-N-SH cells as a percentage of control clonogenicity after exposure to etoposide for varying intervals.

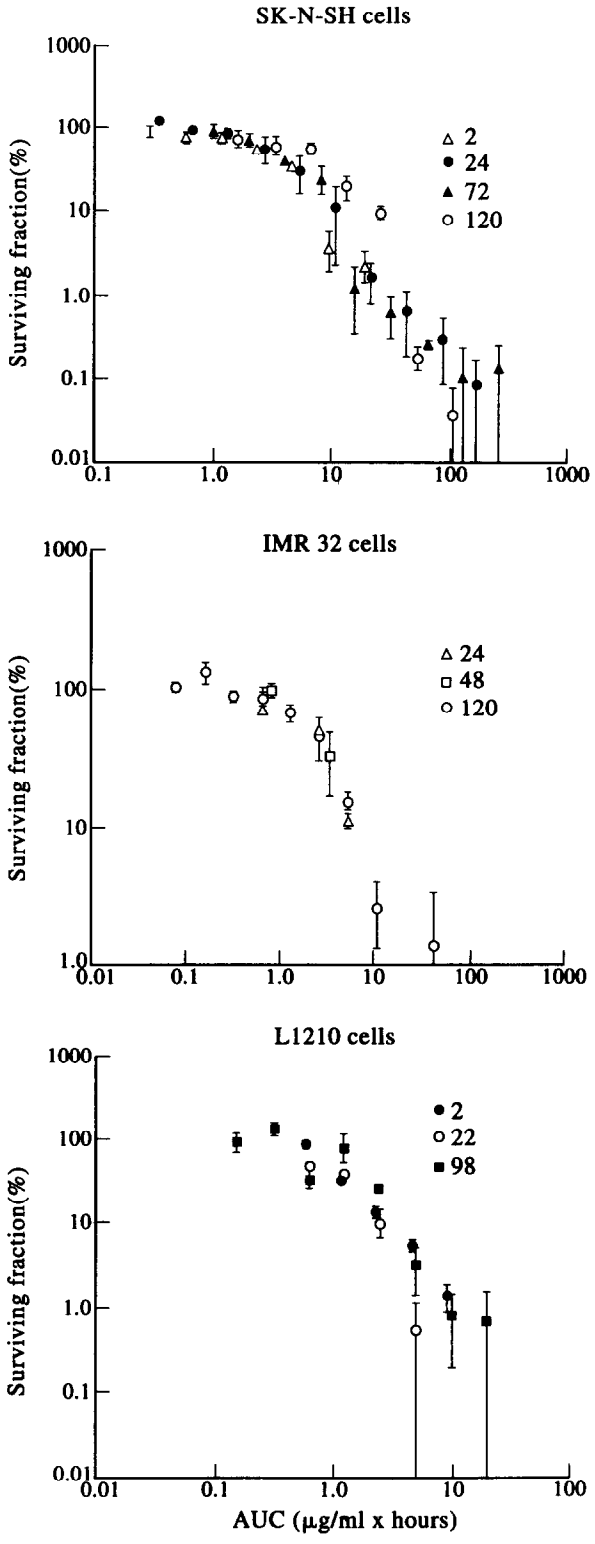

Figure 3. The relationship of the surviving fraction of cells to exposure to etoposide for each cell line. Points refer to mean of at least 3 values ± 1 standard deviation.

Table 1. The relationship of the surviving fraction of SK-N-SH cells to total exposure for continuous and intermittent exposure

	Duration of exposure (h)					
	2	1 × 2	4	2 × 2	48	2 × 24
AUC 50	2.0	2.0	3.3	3.9	3	2.1

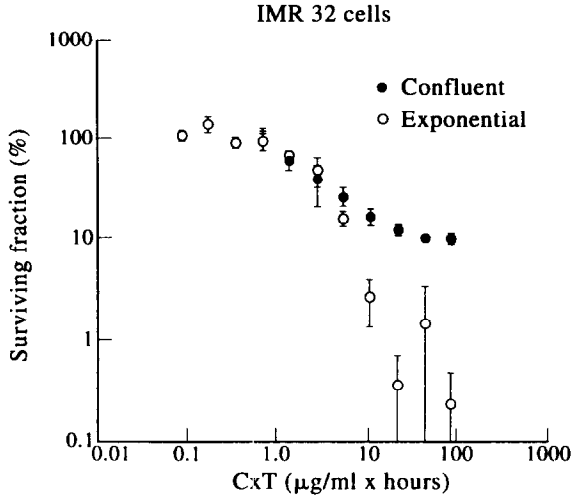

Figure 4. The surviving fraction of IMR 32 cells after exposure to etoposide when in exponential growth and at confluence.

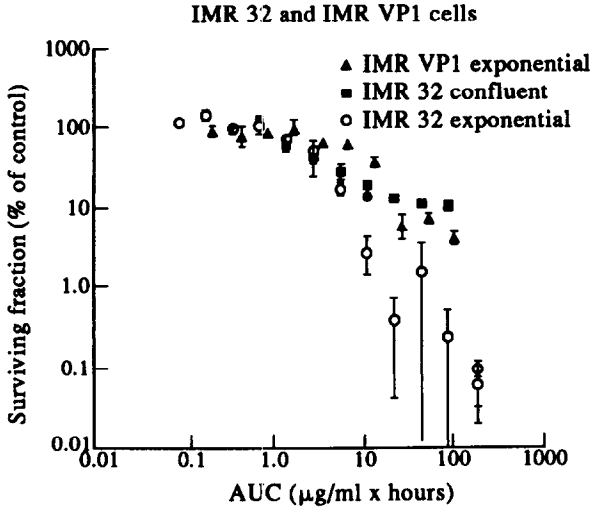

Figure 5. A comparison of the surviving fraction of IMR VP1 cells in exponential growth with IMR 32 cells after exposure to etoposide.

hence increased cytotoxicity with equivalent exposure. In our study, however, no difference in cytotoxicity was seen in exponentially growing cells following equivalent exposures between 2 and 120 h. The presence of a significant quiescent population of cells *in vivo*, but not *in vitro* may be assumed for neuroblastoma cells, but is unlikely to be the case for the transformed murine leukaemia cell line L1210, in which the estimated population

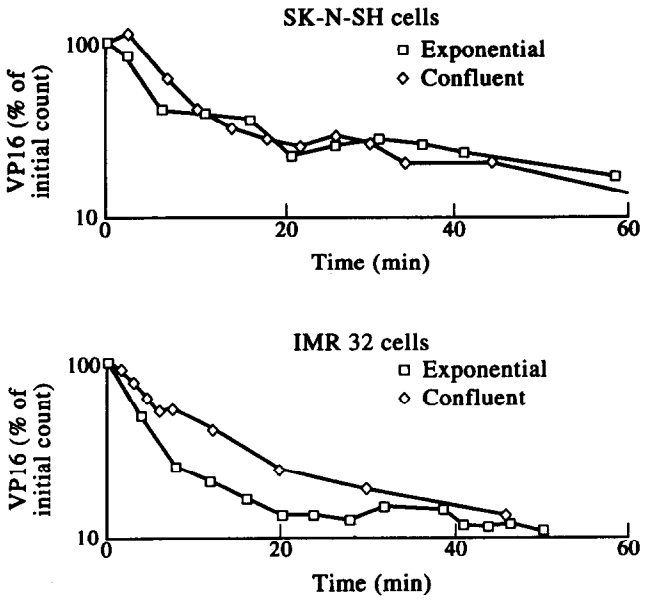

Figure 6. Efflux of radiolabelled VP16 from SK-N-SH and IMR 32 cells in exponential and confluent phases of growth. All experiments were performed at 25°C.

doubling time *in vivo* is 18 h. This would suggest that recruitment is *not* likely to be the mechanism leading to enhanced cytotoxicity and schedule dependency of etoposide *in vivo*.

Schedule dependency of chemotherapeutic agents is well recognised, and forms the basis for more rational therapeutic regimens in many tumours. It is often assumed that cell cycle dependent agents, such as methotrexate, will be schedule dependent, whereas cell cycle independent agents, such as cisplatin or alkylating agents, will not, and cell cycle specific action has been taken to explain *in vivo* findings. The data presented in this paper suggest that, despite evidence for optimum schedules of administration in murine and human subjects, no such schedule dependency exists for etoposide *in vitro*. By implication, cell cycle specificity alone cannot be the explanation for schedule dependency, and some other factor is required.

The effects of duration and concentration of exposure to etoposide upon cytotoxicity *in vitro* have been studied in several systems using either clonogenic assays or cell counting methods after various intervals of exposure [13–17]. In the study by Roed and associates greater cytotoxicity with prolonged exposure to etoposide was taken to imply schedule dependency, although the conditions used in these experiments make an accurate estimate of total exposure impossible. The majority of studies are supportive of the data presented in this paper, and suggest that schedule dependency, where found, is not explained by cell cycle specificity, but is likely to relate to host and tumour cell parameters. For example, administration of etoposide over varying durations or in differing schedules will lead to equivalent total patient exposure as measured by the plasma etoposide AUC, but may be associated with significant differences in distribution of etoposide. In particular, prolonged administration may be associated with greater tissue exposure to etoposide, and hence greater cytotoxicity within this compartment may be seen. Alternatively, where intracellular metabolism of a drug is important in modulation of activity, as for methotrexate,

administration over differing schedules may lead to differing effects. In either case, it is the interaction of host factors, such as drug pharmacokinetics and administration schedule, with tumour cell parameters which is likely to lead to variable effects.

The presence of a resistant subpopulation of cells at confluence is possibly associated with a greater proportion of cells in G0. Flow cytometry of IMR 32 cells at confluence or in exponential growth shows a higher proportion of cells in G0/G1 phase (69 versus 49%), and a smaller proportion in S phase (17 or less versus 40%, data for IMR 32 cells). Quiescent cells would be expected to express low levels of topoisomerase II, in particular the alpha isoform, and since this is the target enzyme of etoposide, a degree of resistance may arise. Topoisomerase II activity may also be modified by, for example, phosphorylation, and studies of both topoisomerase II expression and activity are in progress.

Neuroblastoma cells have also been reported to express P-glycoprotein at higher levels as they undergo differentiation [21 and reviewed in 22]. Both SK-N-SH and IMR 32 cells show increased neurite outgrowth as they reach confluence, and this differentiation may, therefore, be associated with a transient increased resistance. Quantitation of cellular P-glycoprotein levels is currently being performed, and results will be published at a later date.

It is perhaps not surprising that drug efflux studies failed to demonstrate any difference between IMR 32 cells at confluence or in exponential growth. If the resistant subpopulation of cells identified at confluence comprises approximately 15% of the total, and these were to express perhaps 10 times more P-glycoprotein, then the whole population expression would rise by only 2.4-fold, which may not lead to a detectable change in drug efflux.

In conclusion, these studies have shown that etoposide does not demonstrate an optimum concentration or duration of exposure for cytotoxicity of neuroblastoma cells *in vitro*. Exposure to etoposide on separate occasions leads to cytotoxicity which is equivalent to that with continuous exposure. Neuroblastoma cells at confluence develop a subpopulation of cells which is highly resistant to etoposide despite no previous exposure to the drug. Finally, we would suggest that schedule dependency *in vivo* may be related to host pharmacokinetic factors in addition to tumour specific factors.

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