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ORIGINAL ARTICLE

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The schedule-dependent effects of etoposide in leukaemic cell lines: a function of concentration and duration

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Abstract *Purpose*: Etoposide is a commonly used anticancer agent that is highly schedule-dependent. The in vitro activity of etoposide (0–10 μ*M*) was investigated in a panel of leukaemic cell lines. *Methods*: Cells were cultured with etoposide in drug schedules of equal exposure duration (ED, duration×concentration), and the effects of drug exposure on cell parameters, including cell cycle distribution, were assessed over an 8-day period. *Results*: Proliferation assays indicated a concentration- and duration-dependent induction of cell death by etoposide in CEM and HL60 cells, and flow cytometric analysis indicated that this cell kill was by apoptosis. Efficacy was also dependent upon schedule, with more cell kill seen in schedules of longer duration. As an example, accumulative percent cell kill resulting from a continuous exposure to 0.05 μ*M* etoposide was significantly greater than that in cultures involving either a 4-day exposure to 0.1 μ*M* or a single-day exposure to 0.4 μ*M* etoposide ($193.4 \pm 15.9\%$ vs $125.2 \pm 5.4\%$ vs $42.3 \pm 5.9\%$, respectively; $P < 0.001$ in all cases; equi-ED 0.4 μ*M*.days). Efficacy was also dependent upon the ED of the schedule. At very low concentrations, the initial enhancement of cytotoxicity mediated by increasing duration would gradually and paradoxically be lost in the more protracted schedules (e.g. accumulative percent cell kill $66.4 \pm 7.4\%$, $158.3 \pm 12.0\%$ and $40.1 \pm 6.0\%$ with 100 n*M* for 2 days, 33 n*M* for 6 days and 25 n*M* for 8 days, respectively; $P < 0.001$ in all cases; equi-ED 0.2 μ*M*.days). *Conclusions*: Our results confirm the schedule-dependency of etoposide in vitro, highlighting the importance of total duration of drug exposure in determining cytotoxicity, and emphasizing the requirement to achieve a cytotoxic concentration in longer exposures. It is therefore crucial to ensure that etoposide regimens used clinically involve doses that are effectively cytotoxic.

Keywords Etoposide · Drug schedule · Cell viability · Population doubling · Sublethal

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Introduction

Etoposide is a podophyllotoxin derivative that has found wide use as a first-line agent in a variety of neoplasms. It is used extensively in the treatment of solid tumours, particularly small-cell lung cancer, where it remains one of the most active agents both singly and in combination [1]. Etoposide has also been studied in the treatment of a variety of adult and childhood leukaemias, particularly acute myeloid leukaemia (AML), in which its activity has been shown in patients both as a single agent and in combination with other active agents such as cytarabine and the anthracyclines [2]. Besides its use in AML, it has also been employed in combination with other active cytotoxic agents and growth factors as a treatment for chronic myeloid leukaemia [3].

Etoposide is a schedule-dependent agent, which has shown good efficacy in longer treatment programmes [4, 5]. In an attempt to further improve the drug effect, prolonged exposure to low daily doses of the drug has been investigated in a number of clinical studies, and good activity in a number of tumour types has been shown using these schedules [6]. However, there is still sparse in vitro data comparing prolonged schedules with the shorter schedules of 3–5 days that are typically used, and those that are available are inconsistent. Consequently, the possible benefits of prolonged scheduling over standard regimens have not been completely investigated and elucidated. Indeed, an improved understanding of the cellular effects of etoposide in these clinically relevant schedules would provide useful and supportive data in helping to optimize treatment regimens. This becomes particularly pertinent considering

numerous reports over the past 10 years highlighting the potential danger of protracted administration of etoposide in inducing secondary leukaemias. Although there are conflicting reports as to whether or not the risk of developing secondary leukaemia is dependent upon cumulative dose, a recent review by the National Cancer Institute showed it to be a factor of lesser importance [7]. Instead, rather than being dose-dependent, the risk is more dependent on drug schedule and increases in more protracted schedules.

The aims of this study were to investigate the relationship between etoposide exposure schedule and the induction of cytotoxicity in leukaemic cell lines.

Material and methods

Cell culture

CEM (acute lymphoblastic leukaemia), HL60 (acute promyelocytic leukaemia) and K562 (chronic myeloid leukaemia) cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, in a humidified atmosphere of air containing 5% CO_2 at 37°C.

The effect of a continuous 5-day exposure to etoposide was investigated by culturing cells (2×10^5 cells/ml) with etoposide (0–10 μM). Drug and medium were replenished daily because of the limited stability of etoposide in culture medium [8]. For the assays investigating the effect of schedule, only CEM and HL60 cells were used, and treated according to one of numerous 8-day schedules. These were divided into two groups of schedules, with equivalent exposure duration (ED) of either 0.2 or 0.4 μM.days. An example of such schedules in the 0.4 μM.days group consisted of culturing cells with 0.4 μM etoposide for 1 day followed by culture in drug-free medium for the remaining 7 days, 0.2 μM for 2 days then drug-free for 6 days, 0.1 μM for 4 days then no drug for 4 days, 0.067 μM for 6 days then no drug for 2 days, and 0.05 μM for 8 days. These EDs were selected on the basis of their ability to induce cell kill as reported previously [9]. Aliquots were removed daily for cell counts and assessment of viability by trypan blue dye exclusion, and cell cycle distribution by flow cytometry.

Flow cytometric analysis of the cell cycle

The distinct phases of the cell cycle, including the apoptotic fraction, were classified by DNA staining with the fluorescent dye propidium iodide (PI). These were measured by flow cytometry according to methods described previously [10]. Acquisition of data was performed within 1 h using a Becton Dickinson FACSCalibur (BD Biosciences, Oxford, UK), and gating was employed to remove doublet artefacts and to discriminate cells from debris. For each data point, 5000 cells were analysed, and the percentages of cells in sub-G_1 (apoptotic fraction, cells with a reduced PI stain but similar morphology), G_1, S and G_2/M phases were determined using the cell cycle analysis program CellQuest v3.4.

Statistical analysis

All statistical analysis was carried out using Minitab version 13 (State College, Pa.). Control samples were normally distributed as determined by the Shapiro-Wilk test, and parametric tests were used throughout. Any differences between test samples and control cultures, as determined by ANOVA, were further characterized by the standard paired Student's *t*-test.

The concentration of etoposide causing a 50% reduction in cell viability was determined with an adapted version of the sigmoid E_{max} model:

$$E_P = E_C - \left(\frac{E_{\max} \times C^n}{IC_{50}^n + C^n}\right)$$

where E_P is the predicted effect, E_C the control effect, E_{max} the maximum effect, C the concentration of drug, and n the sigmoid-fit factor [9].

Results

Control cultures of all CEM, HL60 and K562 cell lines followed the normal growth pattern with a doubling time of 24–30 h with minimal loss of percentage viability (%V) over the 5-day culture (%V $>95\%$ at all time-points). Control cell cycle distributions for each cell line were: CEM, 1% apoptotic, 39% G_1, 31% S, 26% G_2/M; HL60, 4% apoptotic, 47% G_1, 24% S, 23% G_2/M; K562, 2% apoptotic, 42% G_1, 29% S, 25% G_2/M.

Continuous exposure

Results showed etoposide to be an effective cytotoxic agent in both CEM and HL60 cells, with complete ablation of cell numbers at concentrations >0.8 μM over 3 days. In the presence of drug, cell numbers and percentage viability decreased in a concentration- and duration-dependent manner. The concentrations of etoposide resulting in 50% reduction in percentage viability on day 5 (IC_{50}) were calculated to be 0.39 μM and 0.15 μM for CEM and HL60, respectively. Figure 1 shows the concentration- and duration-dependent reduction in both percentage viability and cell numbers in CEM and HL60 cells.

Flow cytometric analysis of both CEM and HL60 cells revealed significant changes in the DNA profile following treatment with etoposide. The results for both cell lines were similar, so results from CEM alone are presented here, and emphasize both the concentration- and duration-dependent nature of the drug (Fig. 2). After a 24-h exposure, cells showed a concentration-dependent block at the G_2/M phase of the cell cycle, which was mirrored by a decreasing number of cells in the other phases, particularly in the G_1 phase. This block in the G_2/M phase was transient, since continued exposure to higher concentrations of etoposide resulted in an emptying of cells from this phase (percentages in G_2/M among cells with 0.4 μM on days 1, 3 and 5 were 62.1 ± 2.1, 40.3 ± 6.4 and 12.3 ± 1.2, respectively). Importantly, the release of cells from G_2/M was associated with an increase in apoptosis, as indicated by an increase in the sub-G_1 population (percentages of apoptosis among cells with 0.4 μM on days 1, 3 and 5 were 11.5 ± 1.8, 37.5 ± 6.5 and 75.7 ± 1.0, respectively).

In contrast to CEM and HL60 cells, K562 cells were resistant to etoposide-induced cell kill even at the highest

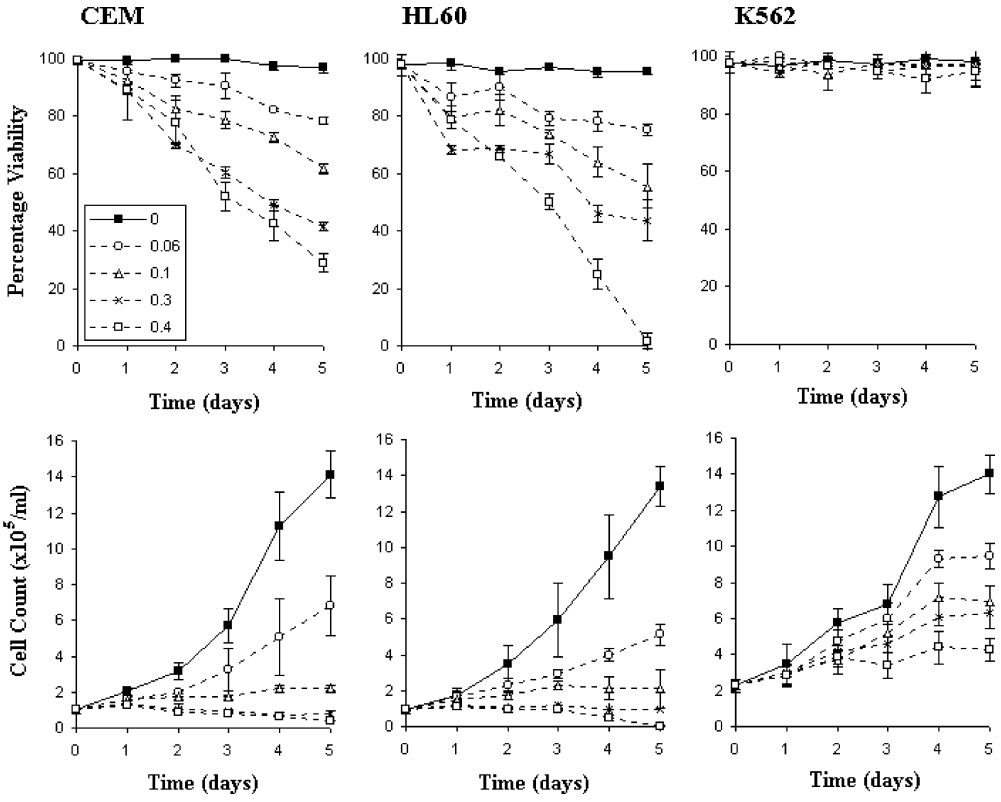

Fig. 1 Effect on percentage viability and cell number in CEM, HL60 and K562 cells of continuous treatment with etoposide (0–0.4 μ*M*). Data points are the means and SDs of three separate experiments

concentration and longest duration (%V >90% at 10 μ*M* on day 5). Instead, cells responded by undergoing cytostasis as indicated by a block in the cell cycle and a reduction in the apparent rate of cell proliferation, coupled with no loss of percentage viability (Fig. 1).

The effect of schedule

CEM and HL60 cells were exposed to etoposide according to a number of schedules that involved culture for 8 days. Thus cells exposed to drug for less than 8 days were then maintained in drug-free medium to ensure that the schedules lasted exactly 8 days. The results indicated that total cell viability and cell growth varied according to the schedule used, and more specifically, that the duration of drug exposure and the total ED were both crucial factors in determining efficacy.

Schedules with EDs of 0.4 μ*M*.days

Significantly greater cytotoxicity was achieved in schedules involving longer exposures compared to those with a short exposure, even at the same ED. This was most evident in the 0.4 μ*M*.days ED group, when comparing percentage viability and percentage apoptosis on day 8 in CEM cells exposed to 0.4 μ*M* etoposide for just 1 of the 8 days with a schedule involving culture with 0.05 μ*M* etoposide for the entire 8 days. Although

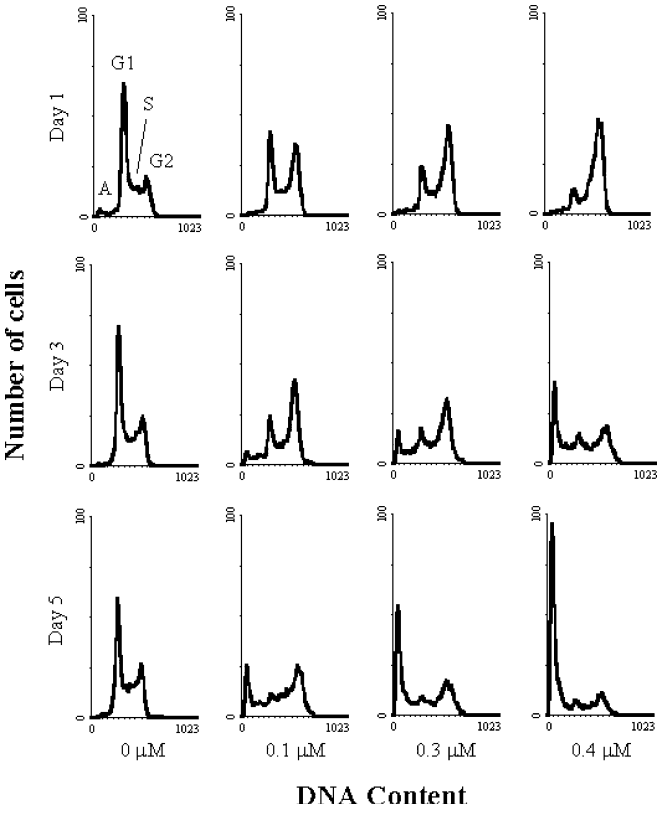

Fig. 2 Effect on cell cycle parameters in CEM cells of continuous treatment with etoposide (0–0.4 μ*M*). Histograms are representative of three separate experiments (*A* apoptotic population)

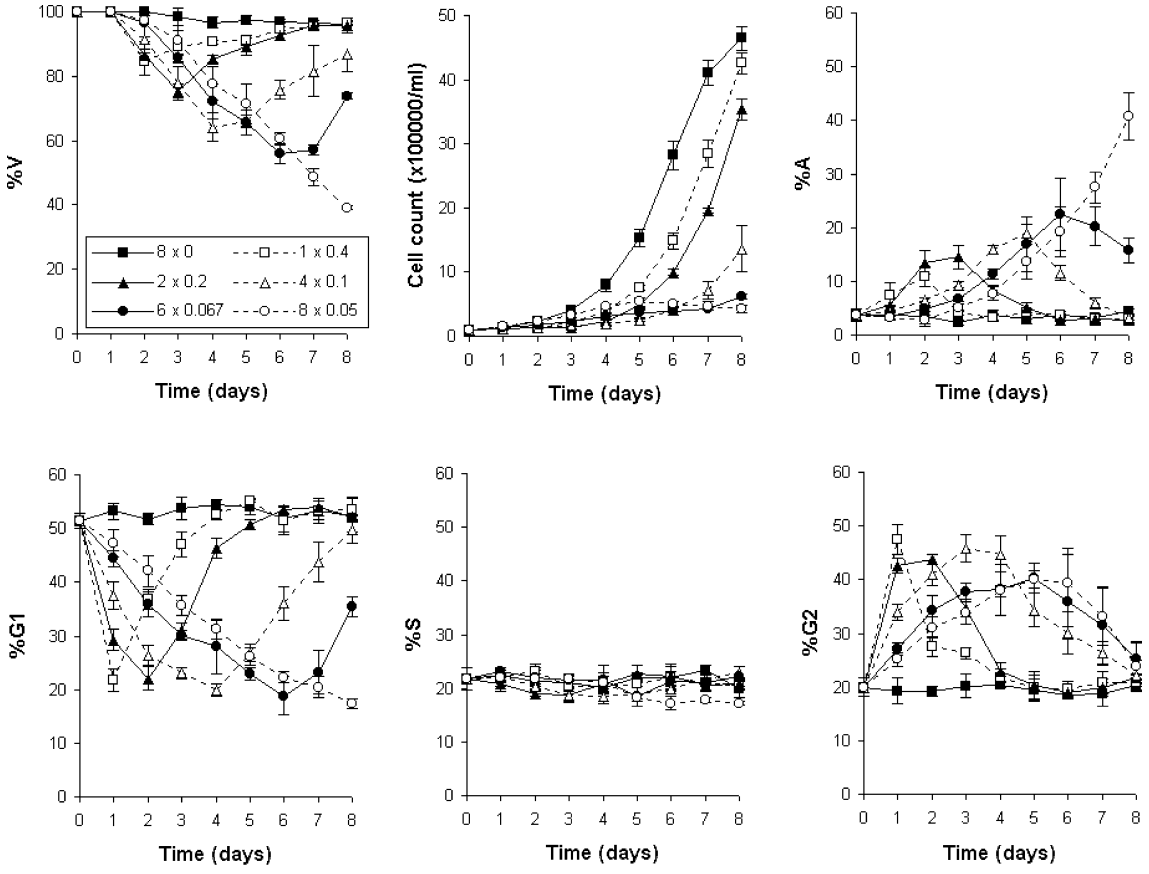

Fig. 3 Effect of five equi-ED schedules (0.4 μ*M*.days) on percentage viability (*%V*) and cell count in CEM cells. The percentage distributions of cells in each phase of the cell cycle, including apoptosis (*%A*), are also shown. Data points are the means and SDs of three separate experiments

the ED in both cases was identical, there was a significantly lower percentage viability ($39.1 \pm 0.5\%$ vs $96.7 \pm 0.7\%$) and more apoptosis ($40.8 \pm 4.3\%$ vs $2.9 \pm 0.9\%$) in the more extended schedule (both $P < 0.001$; Fig. 3). In addition, the total number of surviving cells was lower in those schedules involving longer drug exposure. Comparison of cumulative percent apoptosis and percent cell kill in all these schedules revealed higher levels of both in the protracted schedules at lower etoposide concentrations compared to the shorter schedules at higher concentrations (cumulative percent apoptosis: $39.8 \pm 4.1\%$, $76.5 \pm 4.0\%$ and $101.3 \pm 8.9\%$ in the schedules 1×400, 4×100 and 8×50, respectively; $P < 0.001$; Fig. 4a).

Schedules with EDs of 0.2 μ*M*.days

Drug efficacy was also dependent upon the overall ED. If this was below an effective threshold, there was a gradual reduction in drug efficacy in schedules of more protracted duration (7 days or more). This was clearly highlighted by comparing cumulative percent cell kill in two schedules of equi-ED (0.2 μ*M*.days), one involving 29 n*M* for 7 days and the other 33 n*M* for 6 days ($83.9 \pm 11.2\%$ and $158.3 \pm 12.0\%$; $P < 0.001$; Fig. 4b).

Discussion

This study was undertaken to investigate the effect of schedule on etoposide activity in leukaemic cell lines. In addition to confirming that etoposide was able to reduce percentage viability in cells in a concentration- and duration-dependent manner, we showed through comparative analysis of drug schedules of equi-ED that a higher level of cell kill was generally achieved in schedules of longer duration. However, supplementary to this was the prerequisite of using a sufficiently potent ED to ensure effective cell kill, as highlighted by the reduced/lack of cytotoxicity in the longer 7- and 8-day exposures in the 0.2 μ*M*.days schedule cohort.

Initially, three cell lines were chosen for our studies, but etoposide-induced cell kill was minimal in the K562 cell line, even after culture with super-concentrations (> 10 μ*M*). This observation is in agreement with existing reports detailing drug-resistance in this cell type [10, 11], and since one of the aims of our study was to investigate the effect of drug efficacy in cell lines sensitive to the drug, this cell line was omitted from the drug schedule experiments.

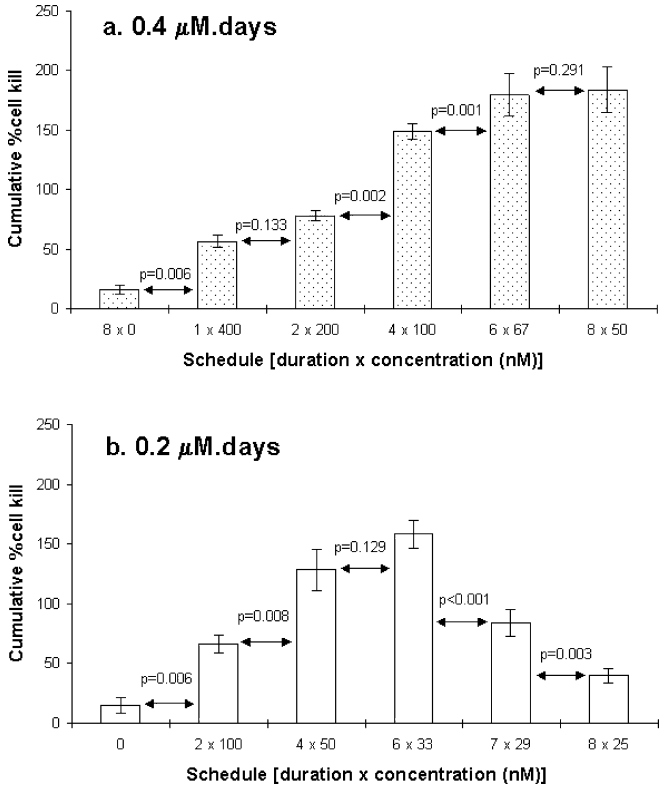

Fig. 4a, b Total/cumulative percent cell kill on day 8 was calculated in CEM cells cultured in two groups of schedules of equi-ED: (**a**) 0.4 μ*M*.days or (**b**) 0.2 μ*M*.days. Data points are the means and SDs of three separate experiments

The target of etoposide action is the nuclear enzyme topoisomerase II (topo II). Mechanistically, etoposide stabilizes the interactions between DNA and topo II, which results in chromosomal aberrations and double-stranded breaks [12, 13]. This DNA damage, which is inextricably linked to the process of DNA replication, occurs in a concentration- and duration-dependent manner, as highlighted by our initial continuous-exposure experiments, which showed clear duration- and concentration-dependent increases in cell kill. We next investigated the effect of schedule on drug efficacy by comparing cell kill in 8-day schedules of equi-ED. The EDs were selected on the basis of their cytotoxic potential: 0.2 μ*M*.days was suboptimal and 0.4 μ*M*.days was optimal [9]. First, the comparison of the schedules of 0.4 μ*M*.days revealed greater loss of viability in the schedules of longer duration, which suggested that the duration of exposure to etoposide was just as important as the exposure concentration in determining etoposide activity. As the length and quantity of S-phase transitions (DNA synthesis) influences etoposide efficacy [14], cultures involving exposure to etoposide for shorter periods of time would have fewer cells that had completed a full cell cycle than those involving exposure to etoposide for a longer duration. The overall ED was also of crucial importance, as we showed that an ED of 0.2 μ*M*.days was less active than the 0.4 μ*M*.days counterpart. Paradoxically, rather than an expected enhancement of cytotoxic effect, there was a gradual loss of cytotoxicity with increasing duration in the 0.2 μ*M*.days cohort of schedules. It was likely that with the prolonged exposures (29 n*M* for 7 days and 25 n*M* for 8 days) the concentration of etoposide on individual days was not sufficient to induce lethal damage.

A similar approach to investigating the effect of exposure and scheduling on etoposide efficacy was employed by Lowis et al. [15], who measured drug effect by clonogenic assays. In contrast to our results, they concluded etoposide cytotoxicity to be independent of schedule, and related only to total drug exposure. Methodologically, viability was assessed by clonogenic and MTT assays. Unfortunately, their analysis only considered the clonogenicity of cells surviving the initial treatment schedules, and the cell viabilities after liquid culture were not established or reported. Therefore, the absolute extent of cell kill caused by the schedules per se was not known [16, 17]. Therefore we utilized a combination of a dye exclusion assay to establish cell viability and flow cytometric analysis to measure apoptosis, and showed a clear schedule effect in etoposide efficacy.

A series of clinical studies by our group over the last decade have confirmed the schedule-dependence of the efficacy of etoposide in vivo [4, 18, 19]. The original landmark study of 1989 compared the efficacy of a pair of etoposide regimens of equivalent ED [4], and clearly showed more responders in a 5-day schedule than in a 1-day schedule. Duration of drug exposure was then extended to 15 days in a concentration-controlled trial to further investigate the schedule effect, while maintaining ED [19]. The activity in the 15-day etoposide arm (1 μg/ml) was markedly less than in the 5-day arm (3 μg/ml) in terms of both antitumour activity [response rates, 14% (2/14) vs 58% (7/12); $P = 0.038$] and haematological toxicity [grade 3–4 leucopenia, 2% (1/48) vs 25% (15/60); $P = 0.001$]. These data suggest that an etoposide concentration of 1 μg/ml for this duration was insufficiently active. Indeed, the results of this current in vitro study support this notion, as our results showed that efficacy could be reduced if the ED was too low (0.2 μ*M*.days vs 0.4 μ*M*.days), or if the duration of drug exposure was excessively long (i.e. 25 n*M* over 8 days vs 40 n*M* over 4 days, Fig. 4b). In addition to reduced efficacy, these inappropriate etoposide schedules could have the adverse impact of delivering to the cells a non/sublethal concentration of drug, resulting in cells with partially damaged DNA that could escape cell death. In fact, such an aberrant phenotype with a reduced apoptotic capability has recently been reported, suggesting a possible association between low sublethal concentrations of etoposide and potential development of secondary malignancies [9]. Furthermore, the exposure of cells to sublethal concentrations of drugs is typically the way that resistant cell lines are created.

In conclusion, these results convincingly confirm the schedule dependency of etoposide in two cell lines. The comparison of schedules of equi-ED clearly highlighted

an increased cytotoxic effect with longer duration schedules. However, we also showed that efficacy could be reduced if the ED used was too low. Thus, extrapolating these results to the clinic, the importance of treating patients with etoposide doses that achieve active plasma concentrations, thus reducing the possibility of cells with aberrant phenotypes surviving treatment, is re-emphasized.

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